# DECLARATION OF RODNEY RICHARDS, Ph.D

I, RODNEY RICHARDS, PhD do hereby make the following sworn declaration. All matters contained herein are of my own personal knowledge unless stated as based upon information and belief:

1. I am scientist familiar with the science behind the testing of "Human Immunodeficiency Virus" (HIV). I have never failed to qualify as an expert in any state or federal court. I have never been found guilty of fraud or perjury. A copy of my curriculum vitae is attached hereto as Exhibit 1, which outlines my education, training and background.

2. In 1977, I received an MS in organic chemistry from the University of Denver. In 1984, I received a Ph.D. in chemistry from the University of Colorado. In 1982, I began full-time work for a small start-up biotechnology company called Applied Molecular Genetics, Inc. in Boulder, Colorado. This company eventually changed its name to Amgen, Inc., and is recognized today as the largest and most successful independent biotechnology company in the world. I worked at Amgen for over 13 years, first as a research scientist and, from 1986 on, as a senior research scientist. My responsibilities at Amgen were primarily centered on the development of novel technologies to enhance therapeutic drug discovery and to improve diagnostic testing methods for the detection of infectious agents associated with human disease.

3. In 1983, a co-worker and I designed and synthesized a modified human interferon gene that ultimately resulted in the production of a recombinant protein with novel anti-viral activity. The Food and Drug Administration (FDA) approved this product (Infergen) in 1997 for use as an anti-viral drug in the treatment of Hepatitis C infections. I also supervised the development of several diagnostic technologies, which resulted in a novel diagnostic test for the detection of genetic material known as DNA/RNA, unique to the HIV. This technology proved to be equal in sensitivity and specificity to a competing technology known as the Polymerase Chain Reaction

(PCR), which is used routinely for the determination of what has become known as the "HIV viral load" levels.

4. During the development of this "HIV test," I retained several consultants including Dr. Kary Mullis, who won the Nobel Prize in 1993 for his invention of PCR, and Dr. Peter Duesberg, a noted authority on retroviruses and a tenured professor at the University of California at Berkeley. These two men brought to my attention many unanswered questions regarding the development and significance of "HIV tests."

5. I have actively reviewed the scientific literature concerning HIV science and related areas, with particular focus on the problems associated with HIV diagnostic testing.

6. To date, the vast majority of diagnostic tests approved by the FDA for medical use related to HIV neither claim to, nor even attempt to, confirm the actual presence of HIV in a patient's sample. In fact, none of the 39 HIV-related diagnostic tests or devices currently listed on the FDA's Website claim to confirm the actual presence of HIV in any sample with any degree of stated accuracy.

7. In short, the standard lingo "HIV test," "testing HIV positive," or a "confirmatory test for HIV," is a complete and total misnomer because none of these tests confirm the actual presence of the virus in a patient's sample. As such, all diagnoses of HIV infection in patients are presumptive based on their risk factors, immune status, and clinical condition (actual health) in combination with other indirect diagnostic evidence for either the past or current presence of HIV in a blood, urine, or saliva sample.

8. The so-called "HIV tests," that are approved by the FDA are intended for use in diagnosing the presence or absence of either antibodies to HIV in a sample, or of molecular fragments of HIV in a sample; never the actual virus. Nevertheless, based

on guidelines and recommendations put forth by the CDC in 1987, virtually all diagnoses of HIV infection since that time have been, and still are, based solely on combinations of HIV antibody tests (Exhibit 2).

9. Antibodies are proteins that the body's immune system produces in response to the presence of germs, such as bacteria and viruses, and do not represent the germ itself. In fact, these antibodies assist in the elimination of the germ by binding to it, thereby interfering with its ability to replicate, and marking it for digestion by other cells in the immune system. Furthermore, germ specific antibodies produced in this way remain at detectable levels in the body for several months to several years, even after the complete elimination of the infectious agent from the body. By analogy, footprints from a mountain lion in a forest do not necessarily mean there is a danger. It could mean the lion has gone away or has been captured. Similarly, antibodies to a germ do not mean the germ is present. Indeed, in the vast majority of cases (such as polio, small pox, measles, hepatitis, chicken pox), it means the germ has been neutralized and eliminated from the body.

10. The two primary classes of so-called "HIV tests" for detecting antibodies to HIV are known as the ELISA (or EIA) and the Western Blot (WB). The detection of antibodies to HIV in a sample is intended to aid a physician in their presumptive diagnosis of infection with HIV. But neither test confirms, or is intended to confirm, the actual presence or absence of virus in a sample.

11. According to current CDC guidelines and recommendations, when a blood, urine, or saliva sample from a patient is presented for testing, it is initially screened for the presence or absence of antibodies to HIV using the ELISA test. If positive, it is retested, and if positive again, the probability that antibodies to HIV are present is considered to be high. However, because "non-specific results [false positives] are found commonly when screening tests are used in low risk populations," and "[s]ince the psychosocial and medical implications of a positive antibody test may be

devastating," samples repeatedly reactive on screening assays such as the ELISA should be further tested using an additional, more specific test for HIV antibodies, such as WB (Exhibit 3).

12. The FDA-approved WB kits are intended for use "as an additional, more specific test for HIV-1 antibodies," in persons already found to be repeatedly reactive on screening tests such as ELISA. However, paradoxically, the manufactures of such products inform us that a sample testing positive on both the EIA screening test and the WB can only be "**presumed** to be positive for **antibody** to HIV-1." (**emphasis** added; Exhibits 3, 4, & 5)

13. The reason that manufacturers of these more specific tests can offer no estimate of how probable it is that such a sample has antibodies to HIV, is because there is currently "no recognized standard for establishing the presence or absence of [HIV] antibody in human blood." (Exhibits 6) The manufactures of HIV-antibody tests also emphasize: "A person who has antibodies to [HIV] is **presumed** to be infected with the virus." (**emphasis** added; Exhibits 3, 5, & 6). The reason one can only presume this to be the case is that according to the scientific literature, "there is no 'gold standard' laboratory test that defines the true infection status." (Exhibits 7) Recognizing that a sample testing positive on both ELISA and WB is only presumed to be positive for antibodies, and further presumed to therefore be positive for the virus, the manufacturers of WB tests agree that the best one can conclude from such a result is that it "… **may** indicate infection with the HIV-1 virus" (**emphasis** added; Exhibits 3, 4 & 8).

14. In spite of the fact that a person testing "positive" on both ELISA and WB are only **presumed** to be positive for antibodies to HIV, and therefore further **presumed** to be infected with the virus, which means they **may** be infected with HIV; at the recommendation of the CDC, it is standard practice in the medical community to use such a combination of test results to tell patients that they have with certainty, a

confirmed infection with the virus itself (HIV). Furthermore, this will be the case even if the patient is without any symptoms (asymptomatic) or risks consistent with the possibility of infection.

15. In my opinion, it is more likely than not that the currently accepted practice of telling people that they are infected with a deadly virus that has no cure, based solely on the results of antibody tests, not actual confirmation of the virus, falls well outside of the FDA-approved use of these products and is scientifically unsound. In part, this disparity between the positions taken by the FDA and CDC can be explained by the fact that the FDA functions under a legal mandate to base product approval on reproducible scientific observations demonstrating statistically significant results. The CDC on the other hand, has no such legal restrictions and can therefore base recommendations solely on theoretical considerations, or simply expert opinion.

16. On the surface, the fact that the CDC would recommend the use of antibody tests for the purpose of telling persons that they are with certainty infected with a deadly and contagious virus – when the manufacturers of these tests warn that they cannot be used to establish with any degree of stated certainty that this is the case – may seem so incredulous that it simply cannot be true. This is particularly the case for persons who were young adults in the late 1980s and 1990s who recall reading in reputable organs of the popular press that the tests being used to diagnose infection with HIV are greater than 99.9 percent accurate.

17. In fact, the manufacturers of HIV antibody tests do report measures of accuracy known as **sensitivity** and **specificity** and, in many cases, these reported measures of accuracy are 99.9 percent, or even greater. However, if one reads the package inserts for these test kits carefully, they will discover that these measures of accuracy have nothing to do with whether or not HIV is present. In fact, the vast majority of these reported measures of accuracy refer to how well one test performs as compared to another test already on the market. In other words, these measures of accuracy refer to

the concordance between two tests; namely, how often a new test says a sample is positive when one already on the market says it is positive (**sensitivity**), and how often does this same test says a sample is negative when the one already on the market says it is negative (**specificity**). Given the fact that all HIV antibody tests are based on the same molecular principles and built from the same portfolio of basic molecular building blocks, it is no wonder that there is a remarkable concordance between their outputs. However, even if two different tests demonstrate 100 percent concordance, that does not justify the use of either for purposes other than what they have been validated and approved for. By way of analogy, if two different manufactures were to construct two different timepieces using the same specifications for the gears and wheels, only to put them in different casings, it is very likely that these two clocks would perform quite similarly. However, even if there were greater than 99.9 percent concordance between these two timepieces, one cannot conclude that either clock measures time accurately.

18. Since the first ELISA and WB tests approved by the FDA had no other approved test for comparison, the manufacturers of these tests choose to validate their products according to their ability to distinguish between persons with confirmed clinical AIDS, and healthy blood donors; the former of which have experienced a plethora of infections and illnesses, and the latter of which represent the healthiest of the healthy and are pre-screened to be free of any risk factors or infections. And in fact, these two tests performed well in distinguishing between these two select populations. However, this tells us nothing about the significance of positive test results in persons in the general population without clinical symptoms of AIDS, and who are not routine blood donors.

19. By way of analogy, a simple test of having persons walk the distance of a balance beam may perform remarkably at distinguishing between persons who are intoxicated, and say, young athletic adults who have not been drinking. However, even if a preliminary evaluation of this test revealed that 99 out of a hundred intoxicated

persons fell off the beam (99% **sensitivity**), and only one out of a hundred young athletes fell off the beam (99% **specificity**), one cannot then simply apply this test to persons in the general population and conclude that 99% of all persons who fall off the beam must be drunk. While it may be the case that 99% of all intoxicated persons in the general population who are subjected to this test will fall off the beam; many others may fall off for other reasons: Perhaps they are clumsy, or had a recent ankle injury or knee surgery, or maybe they are blind. It is for this reason that we cannot say with any degree of certainty how likely it is that any given individual who falls off the beam is drunk. Nevertheless, this would still represent an example of an excellent "screening assay" in that 99% of intoxicated persons who participate in the test most likely would fall off. However, in the absence of a follow-up "confirmatory assay" — in this case measuring the actual blood alcohol content in all positives — the majority of persons who fall from the beam (i.e., positive) may actually be sober.

20. Unfortunately, as detailed above, in the case of HIV, there is no confirmatory laboratory test for either antibodies to HIV, or the virus itself. Although — at the recommendation of the CDC — the WB test is utilized as a confirmatory test for HIV, it is unequivocally not. All of the WB tests on the market are approved only as additional, more specific tests for antibodies to HIV. And even when positive, the best one can do is "presume" the sample is positive for antibodies. And even if that presumption were correct, the significance of those antibodies in persons without symptoms is "unknown." In other words, WB cannot even confirm the presence of antibodies, let alone the virus itself. This is why test manufacturers warn that all persons testing positive for antibodies should be referred to a physician, who can, based on risks, symptoms, and other laboratory data, "decide whether a diagnosis of HIV infection is accurate." The true value of the WB test is that, since it is more specific, it can be used to rule out infection, when negative, in persons testing positive on ELISA screening tests.

21. Although The FDA reported that the "significance of antibodies in asymptomatic individuals is not known" (Exhibit 9) and CDC researchers acknowledged by 1986 that "no established standard exists for identifying [HIV] infection in asymptomatic people," (Exhibit 10) they announced that same year that, "**For public health purposes**," all persons testing positive on ELISA and WB "should be **considered** both infected and infective." (**emphasis** added; Exhibit 11) Of the three references put forth to support this conclusion, one states: "... the frequency of virus in antibody-positive persons is yet to be determined" (Exhibit 12); another makes no mention of whether or not antibodies can be used to infer infection Exhibit 13), and the last one makes reference to yet another CDC publication that states, "the proportion of these seropositive [i.e., antibody-positive] donors who have been infected with [HIV] is not known." (Exhibit 14) Even if it were the case that only half, or even a quarter, of persons testing positive on both ELISA and WB were actually infected with the virus, if physicians were to follow the manufactures instructions and simply tell these persons you "may be infected," then those who may be truly infected might continue to engage in, for example, unprotected sexual activities thereby contributing to the further spread of the virus into the general population. As such, if we want to guarantee that none of these persons contribute to the further spread of this virus, it is necessary to ignore the manufacturers' instructions as well as the scientific facts and tell **all** of these persons that they are with certainty infected; even if they have no risks or symptoms to support such a conclusion.

22. By way of analogy, in the interest of public health, it would be best to tell all persons who fell off the balance beam in the above example that they cannot drive. After all, this would guarantee that 99% of the intoxicated persons in the group tested would not be on the road. Many other sober persons who fell off for other reasons (i.e., false positives) would likewise not be on the road. However, in the absence of a confirmatory test, this would be the sacrifice that would have to be made in order to protect the public from intoxicated drivers. Nevertheless, to turn this scenario on its

head and declare all persons who fell of the beam to be unequivocally intoxicated would be absurd and without any scientific merit whatsoever.

23. Regardless, shortly after FDA approval of the first WB test just over a year later in 1987, the CDC published another report that stripped out any reference to public health, and simply declared, "The presence of antibody [to HIV] indicates current infection [with HIV];" this time without making reference to any scientific study or internal document to substantiate this statement. (Exhibit 2)

24. Fourteen years later (2001), the CDC published revised guidelines stating that persons testing positive for antibodies on both ELISA and WB "are considered HIV-positive and indicative of HIV infection." Although the authors of this document emphasize that "approximately 5,000 abstracts were screened and approximately 600 relevant publications were reviewed," along with "approximately 20 previously published CDC guidelines related to HIV," they do not provide any references to substantiate this statement (i.e., that antibodies equal infection). They also note that in cases where evidence to support their recommendations is lacking, "opinion of 'best practices' by specialists in the field have been used." (Exhibit 15) In summary, the position taken by the CDC and the medical community that all persons testing positive for antibodies to HIV are unequivocally infected with HIV is not a position based on scientific evidence; but rather, one taken in the interest of "public health" based on the "opinion" of specialists in the field.

25. In addition to the concerns detailed above, the scientific literature reveals several studies demonstrating false positive HIV-antibody reactions on ELISA and WB as the result of other conditions completely unrelated to HIV. These include conditions such as infection with other viruses and bacteria, autoimmune conditions, lymphoma, dermatologic disorders, vaccination, hepatitis, herpes, alcoholic liver disease, arthritis, and pregnancy to name a few. (Exhibits 16, 17 & 18)

26. Another class of FDA-approved "HIV tests," which detect a molecular fragment of HIV (not the virus) are the so-called Viral Load (VL) tests. Like the antibody tests, VL tests are not intended for use in diagnosing infection with HIV; rather, they are approved for either assisting a physician in offering a prognosis of disease progression, or to assist in the clinical management of medications in persons already deemed to be HIV positive by other means. In fact, the manufacturers of these tests – i.e. COBAS® AmpliPrep and COBAS® TaqMan® HIV-1 Test – explicitly warn that their tests are "not intended for use as a screening test for the presence of HIV-1 in blood or blood products or as a diagnostic test to confirm the presence of HIV-1 infection." (Exhibit 19) The manufacturer of another VL test also emphasizes "The AMPLICOR HIV-l MONITOR Test is not intended to be used as a screening test for HIV or as a diagnostic test to confirm the presence of HIV infection." ( Exhibit 20) The reason VL tests cannot be used to diagnose infection with HIV is because, as highlighted in the literature, "viral load tests for HIV-1 were neither developed nor evaluated for the diagnosis of HIV infection." (Exhibit 21) This is because, as mentioned above, there is no method to establish with certainty which samples are, or are not, truly HIV positive or negative, which could be used in order to establish how well the VL test results might compare.

27. The ultimate standard for proving the existence of a germ is to obtain it in sufficient quantities and purity so that its chemical, morphological, and biological properties can be determined. Given that most germs are present in the body at insufficient levels to achieve this directly, they first have to be "grown" in the laboratory using a process known as "culture." This involves taking a sample from a patient and placing it in an appropriate laboratory environment where the germ can replicate *in vitro* (outside of the body) to levels where it can be purified for characterization. This purification step is necessary in order to ensure that any chemical or biological phenomena observed during characterization is indeed due to the germ, rather than something else, either alone or in combination, that might be in the mixture.

28. Following such characterization of the purified germ, it may no longer be necessary for the scientist to purify the germ from every culture; rather, they can simply look for "phenomena," which have been demonstrated to be unique to the newly characterized germ. For example, the germ may have a peculiar shape that is so unique that the scientist can simply look at some fluid from the culture or directly from the patient under the microscope in order to declare the presence or absence of the germ. Alternatively, the germ may have a unique chemical building block or unique biological activity that the scientist could use as proxy to determine if the germ is present or absent in the culture.

29. Unfortunately, some germs, such as HIV, are so fragile that they have never been obtained in purified form for direct chemical and biological characterization in the first place. As a result, all chemical and biological phenomena said to be due to HIV are necessarily inferred through indirect techniques. As such, while these phenomena are entirely consistent with the presence of HIV, in my scientific opinion, it is impossible to prove they are beyond a reasonable doubt due to HIV, or even that they are unique to HIV. Since the current consensus among virology experts is that a positive culture for HIV is synonymous with infection with HIV, it is reasonable to use HIV culture in combination with other factors to determine if a patient is infected with HIV.

**30.** In my opinion, it is more likely than not that at a minimum, the only valid determination on whether a patient is infected with HIV would be: 1) a positive result from ELISA; 2) a positive result from WB; 3) a positive culture of the virus; 4) risk factors consistent with the possibility of infection; and 5) clinical symptoms characteristic of AIDS that cannot be accounted for by other factors. Anything short of meeting these criteria to determine the HIV status of a patient is speculative and unreliable.

**Roland [redacted]**

31. I have reviewed the more than 4,000 pages of medical records provided in this case (Bates Stamped 619-4922) in an attempt to locate laboratory reports and data relevant to Roland [redacted] HIV status, as well that of his and his ex-wife, Diana [redacted] from the period 2000 to 2012. If I have missed documents (Bates Stamped 1-618), I will have to comment on those at a later date. I have also reviewed the deposition transcript of Mr. [redacted] treating physician, Dr. Sky Blue.

32. I found no laboratory report that indicates that Mr. [redacted] ever tested positive on an ELISA or WB test. I also found no order form; no indication of who may have placed the order, or what kind of sample may have been collected for this testing. I did find notes on a Cumulative Report indicating a sample from the Defendant, collected on 5/12/00 for HIV antibody testing was "SENT TO STATE LAB, RESULTS SENT TO DOCTOR." (Bates Stamped 4851) I also found handwritten notes from Dr. Blue dated May 19, 2000 stating: "HIV EIA [ELISA] equivocal sent to state lab for review." In these same notes he makes no mention of an equivocal WB (Bates Stamped 1801). Four days later (5/23/2000), Dr. Blue also entered notes stating:" State lab Western Blot also equivocal." (Bates Stamped 1800) Finally, there is a Discharge Summary, dated July 19, 2000, (Bates Stamped 1795-1796), in which Dr. Coffman states, "both ELISA and Western blot results were equivocal." Dr. Blue initially testified that he thought Mr. [redacted] initial ELISA test result was "positive," but later amended his testimony to conclude that it was "equivocal" as indicated by his medical records. This is troubling because, according to the manufacturers of ELISA tests and CDC guidelines, there is no such thing as an equivocal ELISA test result.

33. Curiously, there is a lab report, dated 5/26/00, (Bates-Stamped 953) for Mr. [redacted] wife, Diana [redacted] which states that her HIV antibody test result was "nonreactive" or negative.

34. Without a "positive" (sometimes called "reactive") result from an ELISA test and then a WB test, there is no scientific basis whatsoever to conclude that Mr. [redacted] or his wife ever was, or is now, infected with HIV.

35. Dr. Blue testified that he never repeated the ELISA or WB tests on Mr. [redacted] at any time, despite the "equivocal" results.

36. There is a medical note, dated 6/1/00 (Bates Stamped 1800) by Dr. Blue stating that Mr. [redacted] had a VL test result of 410,000. Unlike the ELISA and WB tests, this VL result is supported by a Lab Report, dated 6/1/00 (Bates-Stamped 952). As I explained above in paragraph 26, VL tests, according to their own manufacturers, are not intended to confirm the presence of HIV. These so-called HIV Viral Load tests were approved by the FDA only for use in managing therapy or offering a prognosis of disease progression. The manufacturers of such tests explicitly warn that these tests are "not intended for use as a screening test for the presence of HIV-1 in blood or blood products or as a diagnostic test to confirm the presence of HIV-1 infection"; or "not intended to be used as a screening test for HIV or as a diagnostic test to confirm the presence of HIV infection." As such, results from these tests provide no reliable evidence that can be used to reasonably conclude with any degree of scientific certainty that the Defendant is, or ever was, infected with HIV.

37. Indeed, Dr. Blue testified that of the 1000 to 1500 AIDS patients he has treated in his professional career, only a tiny handful (5 to 10) were diagnosed with AIDS, based on a VL test in the absence of positive ELISA and WB tests. While it is the case that physicians are at liberty to use these tests to inform their clinical practice as they see fit, the practice of using only VL tests to diagnose HIV infection cannot establish with any degree of known scientific certainty whether or not such a diagnosis is accurate. Others have warned against this practice noting, "viral load tests for HIV-1 were neither developed nor evaluated for the diagnosis of HIV infection." (Exhibit 20) The authors of this publication cite three cases in their region over a two-month period

where persons were falsely diagnosed as HIV infected based on positive VL tests in the absence of positive ELISA and WB results.

38. An important paper on VL testing was published by Piatak et al. in the journal *Science* in 1993. In it, Piatak et al. described 66 patients who were thought to be "HIV positive" and who had Viral Loads ranging from as low as 100 to as high as 9.3 million. Amazingly, however, the authors could not culture HIV in 35 of these patients, including some with Viral Loads as high as 191,800, or 664,500 or even 815, 100. (Piatak et al., *Science*, 259: 1749 – 1754 (1993.).)

39. The name "Viral Load" in my opinion is misleading, because it doesn't count actual viruses; but rather tiny fragments of RNA, thought to be unique to HIV. Piatak et al. state: "Circulating levels of plasma virus determined by QC-PCR also correlated with, but exceeded by an average of nearly 60,000 fold …titers of infectious HIV-1." Indeed, a letter to the journal *Nature* by Sheppard et al., stated that Piatak's data suggested that 99.9% of HIV allegedly detected by VL was "non-cultural, suggesting that it was either neutralized or defective" (Sheppard et al, *Nature*, 364:291- 292 (1993). These studies demonstrate that high VL numbers do not necessarily correlate with the actual presence of any infectious virus. As such, the observation that Mr. [redacted] VL levels dropped from 410,000 to undetectable over a three month period while taking antiviral medications doesn't mean he ever had any actual culturable virus in his body.

40. Little is known about the significance of positive VL tests in person who are not also antibody positive because they are rarely ever used for this purpose. In one study that purposely performed HIV VL tests on individuals who were negative for antibodies to HIV, the authors reported false positive rates for three different tests of 6.3, 14.7, and 4.4 percent, respectively. (Sheppard H, et al. JAIDS 1991; 4: 819-823). The observations that Mr. [redacted] had no demonstrable antibodies to the virus and that his CD4 T-cell count has not improved in response to what appears as successful

treatment, suggest Mr. Roland's immunosuppression may be due to something other than HIV.

41. According to his Consultation Notes written on 5/08/2000, Dr. Blue had considered Job's Syndrome (an inherited immunosuppressive syndrome characterized by elevated levels of IgE) as a possible explanation for Mr. [redacted] chronic history of opportunistic infections and skin problems. Dr. Blue noted Mr. [redacted] had, "consistent features: These would include eczema, history of staph skin infections on several episodes. He has frequent allergies, also has sinus respiratory infection history," and that these were "lifelong medical problems." He also emphasized that, "there have been several people in the family who has also been bothered by skin problems, allergies and sinus trouble." Based on this suspicion, an IgE test was ordered on the same day and the results appear to confirm this suspicion. Specifically Mr. [redacted] IgE level was 4,598 with a normal range of 0.5 to 94. A second test ran three days later confirmed this hyper-IgE with a value of 4,979. Doctor Callanan characterized these IgE levels as, "an enormous elevation, the highest that I have personally ever see in a patient;" and that in two studies he read "there was a very minimal elevation in AIDS patients of the IgE levels …" (5/14/2001; Bates Stamped 1454) According to researchers from the National Cancer Institute: "A review of reported cases of lymphoma in Job's syndrome indicates an increase in relative risk of 259 (95% confidence interval 102, 416);" and that: "The cause of the increased risk has yet to be defined but has *similarities to a pathogenetic model of AIDS related lymphoma*." (*Emphasis mine*; Leonard G, et al. Leuk Lymphoma 2004; 45(12): 2521-5.) In his testimony, Dr Blue suggested that these elevated IgE levels were due to Mr. [redacted] lymphoma. However, in his letter written one year later (5/14/2001; Bates Stamped 1454), Dr. Callanan indicates that he enclosed a copy of an article that might "explain [Roland's] hyper-IgE phenomena that we noticed when he first became ill last year."

42. In essence, due to the fact that Mr. [redacted] has no documented risk factors for HIV, did not infect his wife with HIV, did not infect (to my knowledge) either of the complaining witnesses in this case, had only "equivocal" results from the ELISA and WB results (although even this is unconfirmed by any lab reports) and was diagnosed with HIV infection and AIDS based on a Viral Load test, that its own manufacturers state is not intended to be used as a diagnostic test or to confirm HIV infection, it is my opinion, on a more likely than not basis, that there is insufficient evidence to conclude with any degree of scientific or clinical certainty that Mr. [redacted] is, in fact, infected with HIV.

I, RODNEY RICHARDS, do hereby swear under penalty of perjury under the laws of the State of [redacted] that the foregoing, consisting of SIXTEEN of SIXTEEN pages is true and correct.

Executed this fourteenth day of August, 2012 in LOUISVILLE, COLORADO.

RODNEY RICHARDS PH.D.

# ATTACHMENTS

# ATTACHMENT 1

Rodney M. Richards
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## *Education*

PhD in Organic Chemistry, University of Colorado at Boulder. 1978-1982
Title of dissertation: "Synthesis of Molecular Cylinders and Moebius Strips."

MS in Organic Chemistry, University of Denver, Denver CO. 1974-1977
Title of dissertation: "The Reaction of Desyl Chloride with Phenylhydrazine: A Study of Osazone Formation."

BS in Chemistry, University of Denver, Denver CO. 1969-1973

## *Employment/Professional Experience*

Amgen, Inc. Boulder, CO 1982-1995
- Research Scientist (1982-1987)
- Senior Research Scientist (1987-1995)

National Transfer and Register Corp, Westminster, CO 1988-1993
- Board of Directors

Richards Land Management LLC, Louisville, CO 1995-1998
- Owner/Manager

Consultant, Louisville, CO 1996-1997
- Expert witness DNA fingerprinting (forensics)

Independent Research, Louisville, CO 1996-2007
- Computer Modeling (Cellular Communication)
- Community Educator: HIV/AIDS, Hepatitis C
- Expert witness HIV diagnostic testing

Apro Bio Pharmaceutical Corp. Greenwood Village, CO 2007-2008
- Director of Corporate Scientific Operations

Rocky Mountain Biopharmaceutical, Inc. 2008-present
- Co-founder/ Chairman of the Board
- Senior Scientific Director
- Chief Technology Officer

## *Research Experience*

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| Nucleic Acid Synthesis, gene synthesis and assembly, molecular cloning and recombinant protein expression. Designed and synthesized novel Interferon gene, approved by FDA in 1997 for treatment of Hepatitis C infection. | 1982-1984 |
| Developed sensitive non-radioactive nucleic acid clinical diagnostic assays for detection of infectious diseases. | 1984-1988 |
| Developed novel nucleic acid amplification technologies Including a test for HIV DNA equal in sensitivity to PCR. Also developed technologies to eliminate false positive signals. | 1988-1992 |
| Synthesis and screening of combinatorial nucleic acid and protein libraries to assist in drug discovery. | 1992-1995 |
| Independent research scientist: Expert witness DNA forensic and HIV diagnostic testing. | 1995-2007 |
| Director of Corporate Scientific Operations: Coordinated and monitored Corporate sponsored research and intellectual property development at the University of Colorado Denver Health Science Center, Denver Veterans Affairs Medical Center and Colorado State University. | 2007-2008 |
| Co-founder Rocky Mountain Biopharmaceutical, Inc. Currently developing corporate bylaws, business plans, licensing arrangements and intellectual property positions. | 2008-present |

## *Community Activities*

Invited Speaker. A two-day program on AIDS/HIV: Virus theory and the immune system. CUNY Gratuate Center Health Fair. New York City, NY. September 2001.

Invited Speaker. Testing, Testing: A critical look at so-called "HIV" tests. AIDS Reality Check. Los Angeles, CA. October 2001.

Invited Speaker. What you and your doctor need to know about HIV tests. Health Education AIDS Liaison. San Diego, CA. October 2001.

Invited Speaker. What you and your doctor need to know about HIV tests. Alive & Well. Los Angeles, CA October 2001.

Invited Speaker. A Behind the Scenes View: The Struggle To Get An Alternative View On Health Published. Gilpin Street Holistic Center; Denver, CO. November 2001.

Guest speaker. Should You Bet Your Life On an HIV Test? Vital facts every woman needs to know. Women's Aids Reality Movement. San Francisco, CA. March 2003.

Invited Speaker. Viral Disease: From discovery to diagnosis. A community workshop sponsored by The Nature Institute. Ghent, NY. October 17, 2003.

Invited Speaker. Viral Disease: From discovery to diagnosis. A community workshop sponsored by the Holistic Healing Institute, Seattle, WA. November 15, 2003.

Invited speaker. HIV/AIDS: A Deeper Look. A workshop sponsored by Mothering Magazine. Santa Fe, NM. April 2004.

Television Interview. Whats behind the failings in Public Health Policies. "On the Edge" with Jonas Moses. Chicago, Il. May 24, 2008.

Radio Interview. WBAI, New York - 99.5 FM Pacifica Radio – The Caldwell Chronicles, NYC, NY. July 11, 2008.

An interview with Rodney Richards. The Communicators, with Leroy Baylor WHCR 90.3 FM. The Voice of Harlem. Harlem, NY. July 20, 2008

## *Publications*

1. Richards RM. "The reaction of desyl chloride with phenylhydrazine: A study of osazone formation." *A thesis presented to the faculty of the Graduate School of Arts and Sciences of the University of Denver*. Denver, Colorado, 1977.
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# ATTACHMENT 2

Weekly
August 14, 1987 / 36(31);509-515

# Perspectives in Disease Prevention and Health Promotion Public Health Service Guidelines for Counseling and Antibody Testing to Prevent HIV Infection and AIDS

These guidelines are the outgrowth of the 1986 recommendations published in the MMWR (1); the report on the February 24-25, 1987, Conference on Counseling and Testing (2); and a series of meetings with representatives from the Association of State and Territorial Health Officials, the Association of State and Territorial Public Health Laboratory Directors, the Council of State and Territorial Epidemiologists, the National Association of County Health Officials, the United States Conference of Local Health Officers, and the National Association of State Alcohol and Drug Abuse Directors.

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) and related clinical manifestations, has been shown to be spread by sexual contact; by parenteral exposure to blood (most often through intravenous (IV) drug abuse) and, rarely, by other exposures to blood; and from an infected woman to her fetus or infant.

Persons exposed to HIV usually develop detectable levels of antibody against the virus within 6-12 weeks of infection. The presence of antibody indicates current infection, though many infected persons may have minimal or no clinical evidence of disease for years. Counseling and testing persons who are infected or at risk for acquiring HIV infection is an important component of prevention strategy (1). Most of the estimated 1.0 to 1.5 million infected persons in the United States are unaware that they are infected with HIV. The primary public health purposes of counseling and testing are to help uninfected individuals initiate and sustain behavioral changes that reduce their risk of becoming infected and to assist infected individuals in avoiding infecting others.

Along with the potential personal, medical, and public health benefits of testing for HIV antibody, public health agencies must be concerned about actions that will discourage the use of counseling and testing facilities, most notably the unauthorized disclosure of personal information and the possibility of inappropriate discrimination.

Priorities for public health counseling and testing should be based upon providing ready access to persons who are most likely to be infected or who practice high-risk behaviors, thereby helping to reduce further spread of infection. There are other considerations for determining testing priorities, including the likely effectiveness of preventing the spread of infection among persons who would not otherwise realize that they are at risk. Knowledge of the prevalence of HIV infection in different populations is useful in determining the most efficient and effective locations providing such services. For example, programs that offer counseling and testing to homosexual men, IV-drug abusers, persons with hemophilia, sexual and/or needle-sharing partners of these persons, and patients of sexually transmitted disease clinics may be most effective since persons in these groups are at high risk for infection. After counseling and testing are effectively implemented in settings of high and moderate prevalence, consideration should be given to establishing programs in settings of lower

prevalence. Interpretation of HIV-Antibody Test Results

A test for HIV antibody is considered positive when a sequence of tests, starting with a repeatedly reactive enzyme immunoassay (EIA) and including an additional, more specific assay, such as a Western blot, are consistently reactive.

The sensitivity of the currently licensed EIA tests is 99% or greater when performed under optimal laboratory conditions. Given this performance, the probability of a false-negative test result is remote, except during the first weeks after infection, before antibody is detectable.

The specificity of the currently licensed EIA tests is approximately 99% when repeatedly reactive tests are considered. Repeat testing of specimens initially reactive by EIA is required to reduce the likelihood of false-positive test results due to laboratory error. To further increase the specificity of the testing process, laboratories must use a supplemental test--most often the Western blot test--to validate repeatedly reactive EIA results. The sensitivity of the licensed Western blot test is comparable to that of the EIA, and it is highly specific when strict criteria are used for interpretation. Under ideal circumstances, the probability that a testing sequence will be falsely positive in a population with a low rate of infection ranges from less than 1 in 100,000 (Minnesota Department of Health, unpublished data) to an estimated 5 in 100,000 (3,4). Laboratories using different Western blot reagents or other tests or using less stringent interpretive criteria may experience higher rates of false-positive results.

Laboratories should carefully guard against human errors, which are likely to be the most common source of false-positive test results. All laboratories should anticipate the need for assuring quality performance of tests for HIV antibody by training personnel, establishing quality controls, and participating in performance evaluation systems. Health department laboratories should facilitate the quality assurance of the performance of laboratories in their jurisdiction. Guidelines for Counseling and Testing for HIV Antibody

These guidelines are based on public health considerations for HIV testing, including the principles of counseling before and after testing, confidentiality of personal information, and the understanding that a person may decline to be tested without being denied health care or other services, except where testing is required by law (5). Counseling before testing may not be practical when screening for HIV antibody is required. This is true for donors of blood, organs, and tissue; prisoners; and immigrants for whom testing is a Federal requirement as well as for persons admitted to state correctional institutions in states that require testing. When there is no counseling before testing, persons should be informed that testing for HIV antibody will be performed, that individual results will be kept confidential to the extent permitted by law, and that appropriate counseling will be offered. Individual counseling of those who are either HIV-antibody positive or at continuing risk for HIV infection is critical for reducing further transmission and for ensuring timely medical care. Specific recommendations follow:

1. Persons who may have sexually transmitted disease. All persons seeking treatment for a sexually transmitted disease, in all health-care settings including the offices of private physicians, should be routinely * counseled and tested for HIV antibody.

2. IV-drug abusers. All persons seeking treatment for IV-drug abuse or having a history of IV-drug abuse should be routinely counseled and tested for HIV antibody. Medical professionals in all health-care settings, including prison clinics, should seek a history of IV-drug abuse from patients and should be aware of its implications for HIV infection. In addition, state and local health policy makers should address the following issues:

Treatment programs for IV-drug abusers should be sufficiently available to allow persons seeking assistance to enter promptly and be encouraged to alter the behavior that places them and others at risk for HIV infection. Outreach programs for IV-drug abusers should be undertaken to increase their knowledge of AIDS and of ways to prevent HIV infection, to encourage them to obtain counseling and testing for HIV antibody, and to persuade them to be treated for substance abuse. 3. Persons who consider themselves at risk. All persons who

consider themselves at risk for HIV infection should be counseled and offered testing for HIV antibody. 4. Women of childbearing age. All women of childbearing age

with identifiable risks for HIV infection should be routinely counseled and tested for HIV antibody, regardless of the health-care setting. Each encounter between a health-care provider and a woman at risk and/or her sexual partners is an opportunity to reach them with information and education about AIDS and prevention of HIV infection. Women are at risk for HIV infection if they:

Have used IV drugs. Have engaged in prostitution. Have had sexual partners who are infected or are at risk for infection because they are bisexual or are IV-drug abusers or hemophiliacs. Are living in communities or were born in countries where there is a known or suspected high prevalence of infection among women. Received a transfusion before blood was being screened for HIV antibody but after HIV infection occurred in the United States (e.g., between 1978 and 1985). Educating and testing these women before they become pregnant allows them to avoid pregnancy and subsequent intrauterine perinatal infection of their infants (30%-50% of the infants born to HIV-infected women will also be infected). All pregnant women at risk for HIV infection should be routinely counseled and tested for HIV antibody. Identifying pregnant women with HIV infection as early in pregnancy as possible is important for ensuring appropriate medical care for these women; for planning medical care for their infants; and for providing counseling on family planning, future pregnancies, and the risk of sexual transmission of HIV to others.

All women who seek family planning services and who are at risk for HIV infection should be routinely counseled about AIDS and HIV infection and tested for HIV antibody. Decisions about the need for counseling and testing programs in a community should be based on the best available estimates of the prevalence of HIV infection and the demographic variables of infection. 5. Persons planning marriage. All persons considering marriage

should be given information about AIDS, HIV infection, and the availability of counseling and testing for HIV antibody. Decisions about instituting routine or mandatory premarital testing for HIV antibody should take into account the prevalence of HIV infection in the area and/or population group as well as other factors and should be based upon the likely cost-effectiveness of such testing in preventing further spread of infection. Premarital testing in an area with a prevalence of HIV infection as low as 0.1% may be justified if reaching an infected person through testing can prevent subsequent transmission to the spouse or prevent pregnancy in a woman who is infected. 6. Persons undergoing medical evaluation or treatment. Testing

for HIV antibody is a useful diagnostic tool for evaluating patients with selected clinical signs and symptoms such as generalized lymphadenopathy; unexplained dementia; chronic, unexplained fever or diarrhea; unexplained weight loss; or diseases such as tuberculosis as well as sexually transmitted diseases, generalized herpes, and chronic candidiasis. Since persons infected with both HIV and the tubercle bacillus are at high risk for severe clinical tuberculosis, all patients with tuberculosis should be routinely counseled and tested for HIV antibody (6). Guidelines for managing patients with both HIV and tuberculous infection have been published (7).

The risk of HIV infection from transfusions of blood or blood components from 1978-1985 was greatest for persons receiving large numbers of units of blood collected from areas with high incidences of AIDS. Persons who have this increased risk should be counseled about the potential risk of HIV infection and should be offered antibody testing (8). 7. Persons admitted to hospitals. Hospitals, in conjunction

with state and local health departments, should periodically determine the prevalence of HIV infections in the age groups at highest risk for infection. Consideration should be given to routine testing in those age groups deemed to have a high prevalence of HIV infection. 8. Persons in correctional systems. Correctional systems should

study the best means of implementing programs for counseling inmates about HIV infection and for testing them for such infection at admission and discharge from the system. In particular, they should examine the usefulness of these programs in preventing further transmission of HIV infection and the impact of the testing programs on

both the inmates and the correctional system (9). Federal prisons have been instructed to test all prisoners when they enter and leave the prison system. 9. Prostitutes. Male and female prostitutes should be counseled

and tested and made aware of the risks of HIV infection to themselves and others. Particularly prostitutes who are HIV-antibody positive should be instructed to discontinue the practice of prostitution. Local or state jurisdictions should adopt procedures to assure that these instructions are followed. Partner Notification/Contact Tracing

Sexual partners and those who share needles with HIV-infected persons are at risk for HIV infection and should be routinely counseled and tested for HIV antibody. Persons who are HIV-antibody positive should be instructed in how to notify their partners and to refer them for counseling and testing. If they are unwilling to notify their partners or if it cannot be assured that their partners will seek counseling, physicians or health department personnel should use confidential procedures to assure that the partners are notified.

Confidentiality and Antidiscrimination Considerations

The ability of health departments, hospitals, and other health-care providers and institutions to assure confidentiality of patient information and the public's confidence in that ability are crucial to efforts to increase the number of persons being counseled and tested for HIV infection. Moreover, to assure broad participation in the counseling and testing programs, it is of equal or greater importance that the public perceive that persons found to be positive will not be subject to inappropriate discrimination.

Every reasonable effort should be made to improve confidentiality of test results. The confidentiality of related records can be improved by a careful review of actual record-keeping practices and by assessing the degree to which these records can be protected under applicable state laws. State laws should be examined and strengthened when found necessary. Because of the wide scope of "need-to-know" situations, because of the possibility of inappropriate disclosures, and because of established authorization procedures for releasing records, it is recognized that there is no perfect solution to confidentiality problems in all situations. Whether disclosures of HIV- testing information are deliberate, inadvertent, or simply unavoidable, public health policy needs to carefully consider ways to reduce the harmful impact of such disclosures.

Public health prevention policy to reduce the transmission of HIV infection can be furthered by an expanded program of counseling and testing for HIV antibody, but the extent to which these programs are successful depends on the level of participation. Persons are more likely to participate in counseling and testing programs if they believe that they will not experience negative consequences in areas such as employment, school admission, housing, and medical services should they test positive. There is no known medical reason to avoid an infected person in these and ordinary social situations since the cumulative evidence is strong that HIV infection is not spread through casual contact. It is essential to the success of counseling and testing programs that persons who are tested for HIV are not subjected to inappropriate discrimination.

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*"Routine counseling and testing" is defined as a policy to provide these services to all clients after informing them that testing will be done. Except where testing is required by law, individuals have the right to decline to be tested without being denied health care or other services.

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DHHS Department of Health and Human Services

This page last reviewed 5/2/01

# ATTACHMENT 3

AIDS#378

**KIT AVAILABILITY**

## HIV-1 Western Blot Kit

20 Tests

Product Number 72827

U.S. License No. 1133

Manufactured by
**Epitope, Inc.**
Beaverton, Oregon 97008

Distributed by
**Organon Teknika Corporation**
Box 15969
Durham, North Carolina 27704-0969

*For technical assistance, contact Organon Teknika Technical Services at 1-800-682-2666.*

Français

**DISPONIBILITE DU KIT**

## VIH-1 Western Blot Kit

20 Analyses

Numéro du produit: 72827

Numéro de License aux U.S.: 1133

Fabriqué par
**Epitope, Inc.**
Beaverton, OR 97008

Distribué par
**Organon Teknika Corporation**
Box 15969
Durham, NC 27704-0969

France:
Organon Teknika S.A.
5, avenue des Prés - B.P. 26
94267 Fresnes Cedex

Dutch

**VERKRIJGBAARHEID KIT**

## HIV-1 Western Blot Kit

20 Tests

Productnummer 72827

Amerikaanse licentie Nr. 1133

Vervaardigd door
**Epitope, Inc.**
Beaverton, OR 97008

Distributie door
**Organon Teknika Corporation**
Box 15969
Durham, NC 27704-0969

Italiano

**DISPONIBILITÀ DEL KIT**

## Kit per HIV-1 Western Blot

20 Test

Prodotto No. 72827

Licenza U.S. No. 1133

Prodotto da
**Epitope, Inc.**
Beaverton, OR 97008

Distribuito da
**Organon Teknika Corporation**
Box 15969
Durham, NC 27704-0969

Español

**DISPONIBILIDAD DEL BOTIQUÍN**

## HIV-1 Western Blot Kit

20 Exámenes

Producto Número 72827

U.S. License No. 1133

Fabricado por
**Epitope, Inc.**
Beaverton, OR 97008

Distribuído por
**Organon Teknika Corporation**
Box 15969
Durham, NC 27704-0969

Printed in USA PN201-3039 Revision #7



# HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1)

# HIV-1 WESTERN BLOT KIT

**An Enzyme Immunoassay for the Detection of Antibody to Human Immunodeficiency Virus Type 1 (HIV-1) in Human Serum or Plasma.**

For *in vitro* Diagnostic Use

20 Tests

Store at 2-8°C

Western blot

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## NAME AND INTENDED USE

The HIV-1 Western Blot Kit is an *in vitro* qualitative assay for the detection of antibodies to individual proteins of the Human Immunodeficiency Virus Type 1 (HIV-1) in human serum or plasma. It is intended for use as an additional, more specific test for HIV-1 antibodies in persons of unknown risk whose serum or plasma specimens have been found to be repeatedly reactive using screening procedures such as enzyme immunoassay (EIA).

## SUMMARY AND EXPLANATION OF THE TEST

Acquired Immunodeficiency Syndrome (AIDS) is caused by at least two etiologic agents which are designated as Human Immunodeficiency Virus Type 1 (HIV-1) and Human Immunodeficiency Virus Type 2 (HIV-2).[1,2] Infections with HIV-2 are found primarily in parts of West Africa.[2] Current data indicate that HIV infections are transmitted by sexual contact, exposure to blood (including sharing needles and syringes) or certain blood products, and perinatally by mother to infant.[3,4] Many screening EIAs that detect the presence of viral-specific antibody to HIV-1 have been described by investigators.[5] The purpose of these screening assays is to detect potentially infectious units of blood in order to prevent their use in transfusion, or in the manufacture of plasma derivatives. The same assays are used as clinical diagnostic tests.

Patients with AIDS and AIDS-related conditions exhibit a high prevalence of antibody to HIV-1. This antibody has also been found in clinically asymptomatic individuals from whom HIV-1 has been cultured.[6]

Clinical samples have also been described that are reactive in the screening assays but do not contain HIV-1 antibody.[7] Some of these samples possess antibody to certain Class II HLA histocompatibility antigens that are found in some cell lines used to produce the virus. Other persons, who have had no known exposure to HIV-1, produce reactive results in the screening test for still unknown reasons. Such nonspecific results are found commonly when screening tests are used in large populations. Since the psychosocial and medical implications of a positive antibody test may be devastating, it has been recommended that additional testing be performed on such samples to validate the presence of antibody specific to HIV-1.

The Western blot assay, as described by Tsang et al.,[8] is useful for elucidating the specificity of the antibody response to HIV-1. In this assay, inactivated and disrupted proteins of HIV-1 are fractionated by electrophoresis according to molecular weight using a polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS). The resolved protein bands are then transferred to a nitrocellulose sheet. This nitrocellulose sheet is then cut into strips which are reacted with serum specimens. If virus-specific antibody is present, binding occurs in bands corresponding to those produced by viral antigens. These bands are visualized by using a conjugate (goat anti-human immunoglobulin labeled with horseradish peroxidase), followed by a substrate for the enzyme. The presence of HIV-1-specific immunoglobulins in serum specimens is indicated by the labeling of HIV-1-specific proteins on the nitrocellulose strips. Recognized viral antigens produce bands at gp160, gp120, p65, p55, gp41, p31, p24, and p18. (Numbers refer to apparent molecular weight in kilodaltons.)

A sample that is reactive in both the EIA screening test and the Western blot is presumed to be positive for antibody to HIV-1, indicating infection with this virus except in situations of passively acquired antibody or experimental vaccination. Antibodies to HIV-2 may also react with the protein antigens of HIV-1.[9] Therefore, individuals infected

with HIV-2 may have reactive tests in the HIV-1 Western blot assay. Usually, however, the cross-reactivity is incomplete resulting in an indeterminate test result (see Interpretation of Results section). Individuals with positive tests should be referred for medical evaluation. A diagnosis of AIDS can be made only if an individual meets the case definition of AIDS established by the Centers for Disease Control.[10]

## CHEMICAL AND BIOLOGICAL PRINCIPLES OF THE PROCEDURE

The whole cell viral lysate used in the manufacture of the HIV-1 Western Blot Kit is manufactured by Organon Teknika Corporation (U.S. license number 956). It is HIV-1 propagated in an H-9/HTLV-IIIB, T-lymphocyte cell line. It is purified by ultra-centrifugation and inactivated by treatment with nonionic detergent and heat.

When used to manufacture the preblotted strips, inactivated and denatured proteins of the HIV-1 virus are fractionated by SDS-polyacrylamide gel electrophoresis. The resolved protein bands are electrophoretically transferred to nitrocellulose sheets. These preblotted nitrocellulose sheets are cut into strips.

HIV-1 specimens, diluted in Sample Buffer, are incubated with the preblotted nitrocellulose strips. If antibodies to specific HIV-1 proteins are present in a specimen, they bind to epitopes contained in the proteins banded on the strip. Any antibody not bound is removed by washing. The conjugate, peroxidase-labeled goat anti-human immunoglobulin, is then added to the strip and allowed to incubate. It binds to antibodies already bound to viral proteins on the strip. Excess conjugate is removed by washing. The strips are then incubated with Chromagen. The color reaction is stopped by aspiration and washing.

If antibodies to specific HIV-1 proteins (p) or glycoproteins (gp) are present in the specimen in sufficient concentration, brown bands may be visible at one or more of the following positions on the nitrocellulose strip: gp160, gp120, p65, p55, p51, gp41, p31, p24 and p18 (number refers to apparent molecular weight in kilodaltons).

## KIT COMPONENTS SUPPLIED

(20 Test Kit)

**HIV-1 Nitrocellulose Strips** — **20 strips**
Prenumbered nitrocellulose strips, preblotted with resolved HIV-1-specific proteins; packed in a resealable plastic bag between buffer-soaked absorbent paper; buffer contains 0.1% sodium azide as a preservative.

**Chromagen** — **1 bottle; 45 ml**
Phosphate buffered saline containing proprietary chromagen and hydrogen peroxide; contains 0.01% thimerosal as a preservative.

**Powdered Milk** — **1 bottle; 30g**
Non-fat milk solids.

**Sample Diluent Concentrate** — **1 bottle; 100 ml**
Phosphate buffered saline with 3.0% Tween-20; contains 0.01% thimerosal as a preservative.

**Conjugate Concentrate** — **1 vial; 0.1 ml**
Goat anti-human IgG (heavy and light chains) labeled with horseradish peroxidase; contains 0.01% thimerosal as a preservative.





**Negative Control** — **1 vial; 0.2 ml**
Human serum nonreactive for antibodies to HIV-1; tested negative for HBsAg and antibodies to HCV; contains 0.1% sodium azide and 0.01% thimerosal as preservatives.

**Low Positive Control** — **1 vial; 0.2 ml**
Human serum reactive for antibodies to HIV-1; tested negative for HBsAg and antibodies to HCV; heat-inactivated to render material noninfectious for HIV-1; contains 0.1% sodium azide and 0.01% thimerosal as preservatives.

**High Positive Control** — **1 vial; 0.2 ml**
Human serum reactive for antibodies to HIV-1; tested negative for HBsAg and antibodies to HCV; heat-inactivated to render material noninfectious for HIV-1; contains 0.1% sodium azide and 0.01% thimerosal as preservatives.

**Reaction Trays** — **5 each**
Eight-lane disposable trays with lids.

**Caution:** The Negative Control is prepared from human plasma or serum found to be nonreactive for HIV-1 antibodies and tested for Hepatitis B surface antigen (HBsAg) and antibodies to HCV by FDA-licensed methods. The Positive Controls are prepared from anti-HIV-1 positive human plasma or serum, which was heat-inactivated to render it noninfectious for HIV-1. However, as no procedure can offer complete assurance that infectious agents are absent, all specimens of human origin should be considered potentially infectious and handled with care.[11,12]

## EQUIPMENT REQUIRED BUT NOT SUPPLIED

1. Refrigerator (2-8°C)
2. 37°C water bath
3. Graduated cylinders
4. Beaker or appropriate mixing vessels
5. Balance
6. Laboratory timer
7. Magnetic stir plate and stir bar
8. Test tubes: glass, polypropylene, or polystyrene (12x75 mm)
9. Precision micropipets to deliver variable volumes from 5 to 1000 µl
10. Disposable pipet tips
11. Graduated pipets to deliver volumes to 25 ml
12. Scissors
13. Forceps for strip handling (plastic or Teflon-coated)
14. Transfer pipets
15. Rotary platform, capable of rotating at ~50 to 60 rpm (or speed to achieve proper strip motion; see page 6, step 9)
16. Aspiration system
17. Repeating pipet to deliver 2 ml volumes

## KIT STORAGE AND STABILITY

1. Store all components at 2-8°C when not in use.
2. Expiration dates printed on the kit and kit components indicate the limits of stability.
3. Stability of the components after reconstitution or dilution is as follows:
   a. Sample Buffer
      Sample Buffer is held at room temperature while performing assay except

when using overnight sample incubation. During overnight incubation, store Sample Buffer at 2-8°C, and allow it to return to room temperature before use. Discard excess buffer at completion of assay.

b. Sample dilutions
Test tube sample dilutions must be applied to the strips within one hour of dilution. The dilutions are stored at room temperature during that time.

c. Conjugate dilution
Conjugate dilution must be prepared during the last four-minute wash and applied immediately after the wash is complete and the Sample Buffer is aspirated. Excess Conjugate dilution must be discarded.

d. All other kit components are supplied ready to use.

4. Keep strip bag tightly sealed. *Do not let strips dry out.*

## CHEMICAL OR PHYSICAL INDICATIONS OF INSTABILITY

Alterations in the physical appearance of kit materials may indicate instability or deterioration.

**Note:** Sample Diluent Concentrate may contain crystals. This will not affect assay performance if crystals are dissolved before use (see page 6, step 1.a). Chromagen may contain black particulates but their presence does not affect product performance.

## SPECIMEN COLLECTION, STORAGE AND SHIPMENT

1. No special preparation of the patient is necessary. Serum or plasma specimens may be used. No adverse effects were observed using the anticoagulants EDTA, sodium heparin, or sodium citrate. Remove serum from the clot or plasma from the red cells as soon as possible to avoid hemolysis. If necessary, clarify specimens by centrifugation prior to testing. Heat treatment (56°C for 30 minutes) of patient samples is optional. Although results are not typically affected, strip background color may be enhanced in some specimens.
2. Specimens should be free of microbial contamination and may be stored at 2-8°C for up to two weeks. Fresh serum or plasma may be stored long-term at -20°C. Specimens repeatedly frozen or thawed or those containing particulate matter may give erroneous results.
3. Specimens to be shipped must be packaged in accordance with federal regulations governing the transport of etiologic agents.

## PRELIMINARY PRECAUTIONS

1. Keep testing area separate from areas in which blood or blood products for transfusion are stored.
2. Do not pipet by mouth.
3. Do not smoke, eat, or drink while handling test materials.
4. Wear disposable gloves.
5. Handle all materials used in the test including samples, washing solution, reaction trays, and pipets cautiously as though they are capable of transmitting infectious agents.





6. Consult a physician immediately in the event that contaminated materials are ingested or come in contact with mucous membranes or breaks in the skin.
7. Immediately clean up any spills containing potentially infectious material with a 1:10 dilution of ≥ 5% sodium hypochlorite (bleach) and dispose of the cleaning material by an appropriate method.
8. All specimens of human origin should be considered potentially infectious and handled accordingly.[11,12]
9. Dispose of all specimens and material used to perform the test as if they contain infectious agents. Prior to disposal, treat as follows:

| Material | Disposal Procedure |
|---|---|
| Reusable items | Autoclave for 60 minutes at 121°C. |
| Disposable items | Incinerate. |
| Liquid waste | Mix with bleach to yield a final ratio of one part ≥5% bleach to nine parts waste (1:10). Allow the mixture to stand 30 minutes before flushing down the drain. |

## PROCEDURAL NOTES AND PRECAUTIONS

**Note:** FDA has licensed this test kit for use with serum or plasma only. Use of this licensed test kit with specimens other than those specifically approved for use with this test kit may result in inaccurate test results.

1. Do not interchange or combine any kit component, including strips, with components of another kit lot.
2. The Low Positive and Negative Controls must be assayed with ***each*** run. The High Positive must be assayed with the first run of every package of strips, but is optional in subsequent runs. Retain this High Positive Control strip as a reference. It will be compared to the test strips run from that package to determine band identification and placement.
3. It is important that the items used to prepare and dispense Sample Buffer be scrupulously clean (a repeating pipet is preferable for dispensing the 2 ml of Sample Buffer.
4. Prime the pipet tip when measuring samples or reagents.
5. Do not perform the test in the presence of reactive vapors (e.g., from acids, alkalis, or aldehydes), dust, or residual bleach or bleach fumes; the enzymatic activity of the conjugate may be affected or reactivity may be decreased.
6. Avoid contamination of the strips and/or the buffer-soaked absorbent paper in the resealable bag during handling (this may cause false reactivity in subsequent assays).
   a. Prior to removing the strips from the bag, clean the work surface and forceps with isopropyl alcohol.
   b. Change gloves prior to opening the bag.
   c. Always use clean forceps when handling strips.
7. Do not allow strips to dry out prior to sample addition. Place strips into trays only *after* dilutions have been made.

8. **It is essential to avoid cross-contamination between troughs, especially prior to and during sample incubation.**
   a. Add sample to the trough of the reaction tray, using a transfer pipet.
   b. Additional precaution may be taken by placing the strips in every other trough of a tray.
   c. Avoid delivering bubbles to the liquid in the troughs.
   d. As soon as the dilutions have been added to all strips in a tray, cover the tray with lid.
   e Be careful to avoid dislodging fluid from the troughs when transferring trays.
   f. Liquid in the troughs should not contact tray lids.
9. While a rotation speed of 50 to 60 rpm is recommended for each rotation step, this may be adjusted to achieve proper rotation as follows. It is critical that each strip is immersed in the liquid and moves freely; however, liquid must not contact the tray lid during rotation. Monitor assay results for cross contamination.
10. Incomplete or ineffective washing will compromise the assay; it is imperative to follow the wash procedure carefully.
11. Discard used disposable reaction tray as biohazardous waste. Reuse of these trays is ***not*** recommended.
12. Reagents and samples are brought to room temperature (20-25°C) before beginning the assay, except for Conjugate Concentrate and Chromagen, which must both remain refrigerated (2-8°C) until just prior to use. Return all reagents to 2-8°C after use.
13. **Caution:** The Controls, as well as the buffer in the absorbent paper surrounding the strips, contain sodium azide. If discarding into sewer, flush copiously with water. This helps prevent formation of metallic azides which, when highly concentrated in metal plumbing, may be potentially explosive. Decontaminate plumbing periodically according to CDC guidelines.[13]

## HIV-1 WESTERN BLOT TEST PROCEDURE

1. Prepare Sample Buffer as follows:
   a. Check Sample Diluent Concentrate for crystals.
      i. If crystals have formed, dissolve them by warming the entire bottle and its contents in a 37°C water bath for 10 minutes or until crystals are completely dissolved.
      ii. Allow the material to reach room temperature before use.



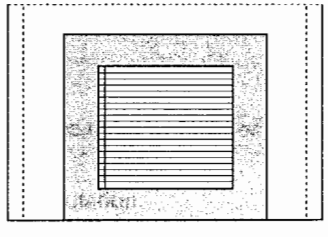


   b. Determine the volume of Sample Buffer to be prepared and quantity of each constituent required from the chart below.

| Total number of strips to be assayed+ | ml of Sample Buffer to prepare* | deionized $H_2O$ required** | ml of Sample Diluent Conc. required** | Powdered Milk required*** |
|---|---|---|---|---|
| 3-6 | 150 | 135.0 | 15.0 | 4.5 |
| 7-9 | 225 | 202.5 | 22.5 | 6.75 |
| 10-13 | 325 | 292.5 | 32.5 | 9.75 |
| 14-16 | 400 | 360.0 | 40.0 | 12 |
| 17-20 | 500 | 450.0 | 50.0 | 15 |

\+ Include strips for controls.
* 25.0 ml of Sample Buffer is required for each strip assayed.
** Sample Diluent Concentrate is diluted 1:10 in deionized water.
*** 3 g of Powdered Milk is required for every 100 ml of diluted Sample Diluent Concentrate.

   c. Combine the required amounts of deionized water, Sample Diluent Concentrate and Powdered Milk.
   d. Mix the solution for a minimum of 15 minutes (ensure the Powdered Milk is completely dissolved).
   e. Store at room temperature while performing assay except when using overnight sample incubation. During overnight incubation, store Sample Buffer at 2-8°C, and allow it to return to room temperature before use. Discard excess buffer at completion of assay.
2. Prepare an assay worksheet, ensuring that the patient sample and control identification is linked to the number embossed on the test strip.
3. Both the Low Positive and Negative Controls must be assayed with ***each*** run. The High Positive must be assayed with the first run of every package of strips, but is optional in subsequent runs. Retain this High Positive Control strip as a reference. It will be compared to the test strips run from that package to determine band identification and placement.
4. Prepare and add controls and patient specimens to nitrocellulose strips as follows:
   a. In appropriately labeled 12x75 mm test tubes, add 40 µl of each specimen or control to 2.0 ml Sample Buffer for a dilution of 1:51. These dilutions must be tested within 1 hour.
   b. Place one prenumbered strip, with *green indicator line facing up*, into each trough as follows, ensuring the strips do not dry out:
      i. For initial use of strips, cut bag below the seal line, keeping upper portion of bag intact, including seal line.
      ii. Cut the lower portion of the bag on the two remaining sides (dotted lines as shown in diagram).
      iii. Fold back the packaging to expose strips.



iv. It is recommended to begin with the left side of the series (strip #1), removing strips to be assayed and placing them in numerical order.

v. Grasp the strip at or above the green indicator line with forceps.

vi. Transfer the strips, avoiding contact with any surface, into the troughs of the reaction tray(s).

vii. Place any remaining strips (still encased in moist absorbent paper and contained in the lower portion of the bag) in the upper portion of bag, seal using zip closure, and return to storage at 2-8°C.

c. Mix and carefully transfer the entire contents of each tube (~2 ml) into the corresponding trough using a transfer pipet.

5. Cover with the tray lid and mix by gentle rotation (~50 to 60 rpm) on a rotator for 10 minutes, ensuring the strips are fully immersed and moving freely in the liquid.

6. Continue incubation using one of the following procedural options:

*Standard:* Incubate at room temperature with rotation (~50 to 60 rpm) for an additional 60 minutes.

*Overnight:* Incubate 16 to 24 hours at 2-8°C without rotation. (After incubation, warm Sample Buffer to room temperature before proceeding.)

7. After incubation, completely aspirate the liquid from troughs (do not allow the strips to dry).

8. Wash the strips immediately as follows:
   a. Add 2.0 ml Sample Buffer to each strip.
   b. Slide the reaction tray(s) back and forth eight (8) times by hand on a flat surface, to achieve a wave motion lengthwise in the troughs. Be sure that each strip is fully immersed in the liquid and moves freely.
   c. Aspirate liquid completely.
   d. Repeat steps a–c two more times.
   e. Add 2.0 ml Sample Buffer to each strip and replace the lid(s).
   f. Place the tray(s) on the rotator (at ~50 to 60 rpm) for four minutes.

9. Prepare diluted Conjugate during the final wash step (step 8.f):
   a. Conjugate dilution must be prepared during the last four-minute wash and applied immediately after the wash is complete and the Sample Buffer is aspirated. Excess Conjugate dilution must be discarded.
   b. Remove Conjugate Concentrate from 2-8°C storage.





c. Determine volumes required of Sample Buffer and Conjugate Concentrate from the following chart:

| Total number of strips being assayed+ | ml of Sample Buffer required* | µl of Conjugate Conc. required** | Total number of strips being assayed+ | ml of Sample Buffer required* | µl of Conjugate Conc. required** |
|---|---|---|---|---|---|
| 3 | 8 | 16 | 12 | 26 | 52 |
| 4 | 10 | 20 | 13 | 28 | 56 |
| 5 | 12 | 24 | 14 | 30 | 60 |
| 6 | 14 | 28 | 15 | 32 | 64 |
| 7 | 16 | 32 | 16 | 34 | 68 |
| 8 | 18 | 36 | 17 | 36 | 72 |
| 9 | 20 | 40 | 18 | 38 | 76 |
| 10 | 22 | 44 | 19 | 40 | 80 |
| 11 | 24 | 48 | 20 | 42 | 84 |

\+ Include strips for controls.

* Prepare 2.0 ml of diluted Conjugate for each strip assayed (a small excess has been incorporated for pipetting ease).

** Conjugate Concentrate is diluted 1:501.

d. Prepare Conjugate dilution by combining required amounts of Conjugate Concentrate and Sample Buffer, and mix well.

e. Return the remaining Conjugate Concentrate to storage at 2-8°C.

10. Aspirate the Sample Buffer completely and add 2.0 ml diluted Conjugate to each trough containing a strip. Replace tray lid.

11. Incubate on rotator (~50 to 60 rpm) for 45 minutes at room temperature.

12. After incubation, aspirate the liquid completely from troughs.

13. Wash the strips as follows:
   a. Add 2.0 ml Sample Buffer to each strip.
   b. Slide the reaction tray(s) back and forth eight (8) times by hand on a flat surface, to achieve a wave motion lengthwise in the troughs. Be sure that each strip is fully immersed in the liquid and moves freely.
   c. Aspirate liquid completely.
   d. Repeat steps a–c three more times.
   e. Add 2.0 ml deionized water to each trough. Replace lids.
   f. Place the tray(s) on the rotator (at ~50 to 60 rpm) for four minutes.

14. During final wash (step 13.f), remove Chromagen from 2-8°C storage and invert bottle two or three times to mix.

15. Aspirate deionized water from each strip.

16. Add 2.0 ml of Chromagen to each strip.

17. Cover tray(s) and gently slide back and forth two to three times by hand on a flat surface.

18. Incubate for 10 minutes at room temperature without rotation.

19. Stop the color development of the strips as follows:
    a. Aspirate Chromagen completely.
    b. Flood the strips with a minimum of 2.0 ml of deionized water.
    c. Let stand for a minimum of 5 minutes.
    d. Aspirate water completely.
20. Allow the developed strips to air dry in tray(s).
21. Handle strips carefully; use clean forceps to remove from troughs.
22. Read and interpret the **dry** strips as soon as possible since developed strips exposed to light may experience fading of bands. Store developed strips away from light at room temperature.

## QUALITY CONTROL

Both the Negative and Low Positive Controls must be assayed regardless of the number of samples tested. A High Positive Control must be assayed with the first run of every package of strips, but is optional in subsequent runs. Retain this High Positive Control strip as a reference. It will be compared to the test strips run from that package to determine band identification and placement.

The following conditions must be met for assay results to be considered valid:

Kit Controls

1. Negative Control: No bands are observed on the strip.
2. Low Positive Control *(Weakly Reactive)*: Bands are present (P) at gp160, gp41 and p24. Other bands may or may not be visible.
3. High Positive Control *(Strongly Reactive)*: Bands are present (P) at gp160, gp41 and p24. Bands are visible at gp120, p65, p51, p31 and p18. The p55 band may or may not be visible (see Figure 1 on page 11).

## INTERPRETATION OF RESULTS

1. Band Identification
    a. Correlate the band position of the High Positive Control strip with Figure 1 on page 11 to identify the HIV-1 viral bands and their positions.
    b. Compare each test strip to the High Positive Control strip for identification of reactive bands.
2. Band Intensity
    a. Correlate the bands of each strip and control to the gp41 band on the Low Positive Control strip and assign a level of intensity as follows:

| Band Intensity | Definition |
|---|---|
| Present (P) | Band intensity is greater than or equal to that of the gp41 on the Low Positive Control strip. |
| Indeterminate (I) | Band is visible but intensity is less than that of the gp41 on the Low Positive Control strip. |
| Absent (A) | No reactivity is observed. |





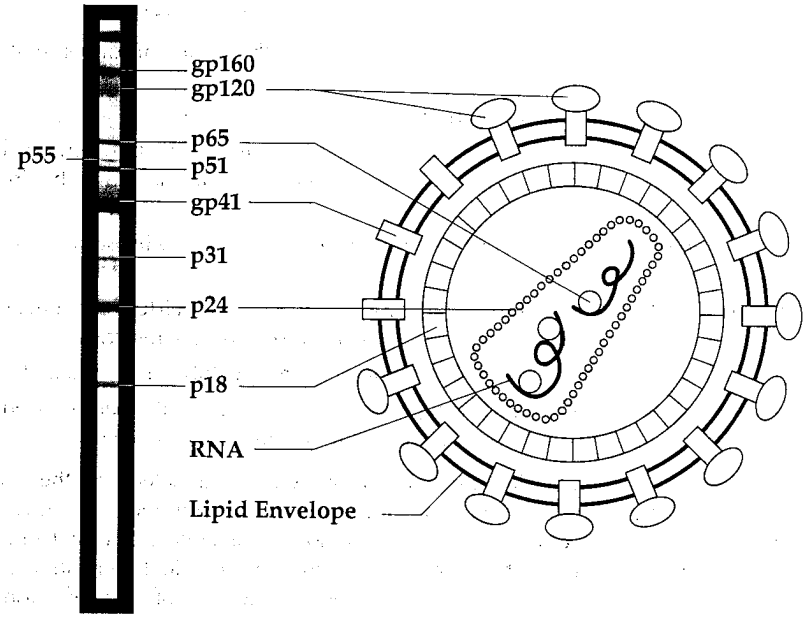
3. Strip Interpretation
    a. Based on band position and reactivity, analyze the results and assign each strip a final result.

| Test Result | Definition |
|---|---|
| Positive | Any two of the three major bands of diagnostic significance must be *Present* (as defined above).<br>(1) gp160 and/or gp120 (2) gp41 (3) p24<br>Other bands may or may not be visible. |
| Indeterminate | Any band reactivity which does not meet the criteria for a Positive result as described above. |
| Negative | Band reactivity is *Absent.* |

**Figure 1: Protein Band Identification on an HIV-1 Western Blot Strip**

On the left is a representation of a Western blot strip developed with High Positive Control. The illustration is a reference for band identification and position (see Interpretation of Results, page 10, step 1).

On the right is a representation of the HIV-1 virus. The bands correlate to corresponding viral subpart origin.



**Viral Origin of HIV-1 Associated Bands**

| Virus Gene | Gene Product and Description | |
|---|---|---|
| *env* | gp160 | env protein precursor |
| | gp120 | outer env protein |
| | gp41 | transmembrane protein |
| *pol* | p65 | reverse transcriptase |
| | p51 | reverse transcriptase |
| | p31 | endonuclease |
| *gag* | p55 | core protein precursor |
| | p24 | core |
| | p18 | core |

## LIMITATIONS OF THE PROCEDURE

1. The assay must be performed in strict accordance with these instructions to obtain accurate, reproducible results.
2. Although a Positive result may indicate infection with the HIV-1 virus, a diagnosis of Acquired Immunodeficiency Syndrome (AIDS) can be made only if an individual meets the case definition of AIDS established by the Centers for Disease Control.[10] A repeat test on an independent sample should be considered to control for sample mix-up or operator error, and to verify a positive test result.
3. Individuals may present incomplete patterns due to the natural history of AIDS or other immunodeficiency states, e.g.:
   a. AIDS patients may lose antibody reactions to p24 & p31;
   b. Infants born to HIV-1 infected mothers, but who are uninfected, may display incomplete patterns as passively acquired maternal antibodies begin to disappear;
   c. Individuals who have recently seroconverted may display incomplete band patterns;
   d. Infected patients with malignancies and individuals receiving immunosuppressive drugs may fail to develop a Positive result;
   e. Individuals infected with HTLV-I/II or HIV-2 may exhibit cross-reactivity;
   f. Individuals may develop incomplete patterns that reflect the composition of experimental HIV sub-unit vaccines that they may have received.
4. A person who has antibodies to HIV-1 is presumed to be infected with the virus, except that a person who has participated in an HIV vaccine study may develop antibodies to the vaccine and may or may not be infected with HIV. Clinical correlation is indicated with appropriate counseling, medical evaluation and possibly additional testing to decide whether a diagnosis of HIV infection is accurate.
5. Since reactivity of any degree with any of the virus-specific proteins present on the strip results in an Indeterminate result, all samples interpreted as Indeterminate should be repeated using the original specimen. In addition, individuals with Indeterminate results should be followed for up to six months.[14]
6. Do not use this kit as the sole basis of diagnosis of HIV-1 infection.





7. A Negative result does not exclude the possibility of HIV-1 infection. Antibody testing should not be used in lieu of donor self-exclusion by blood collection establishments.
8. The HIV-1 Western Blot Kit is a biological product which, although highly consistent, does display variation from blot to blot. Examples of these variations include bands which have slightly wavy or slanted appearance, small artifacts within the banding area, and a light smearing pattern across a set of strips. These are considered normal assay variations which infrequently affect assay interpretation. However, if they do interfere with assay interpretation, call the assay invalid and repeat.

## PERFORMANCE CHARACTERISTICS

### Expected Values

The performance of this HIV-1 Western Blot Kit was evaluated in clinical studies in low risk, high risk, and AIDS populations. Sera were tested with licensed EIA test kits, a previously licensed Western blot kit, and with this kit. A total of 1,026 samples were tested by five independent clinical sites located in both high and low incidence areas. Additional populations obtained for special studies include those described under specific headings.

Specimens from low risk groups including 472 normal blood donors and 23 others of no known risk were collected both prospectively and retrospectively. Of the 345 repository samples reflecting high risk, 29 were from hemophiliacs and 102 were from the cord blood of newborns with mothers at known risk. A study was performed using 51 EIA repeatedly reactive (RR) samples from various geographic locations with high incidence of HIV-1 infection. These were comprised of 12 from Uganda, 12 from Brazil, 14 from Zambia and 13 from the USA. The remaining 163 of the High Risk population includes homosexuals, intravenous drug users (IDUs), prostitutes, sexual partners of persons at risk and those of other acknowledged risk factors. Sera were tested from 186 persons with clinically diagnosed AIDS.[10]

The frequency of virus-specific bands and interpretation by clinical group according to this HIV-1 Western Blot Kit is presented in Table 1.

## Table 1: Frequency of Virus-Specific Bands and Interpretation of Samples Tested by the HIV-1 Western Blot Kit

| Low Risk[a] | HIV-1 WB Result | | Band Specificities (# and % of samples)[d] | | | | | | | | | Non-Viral |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | gp160 | gp120 | p65 | p55 | p51 | gp41 | p31 | p24 | p18 | |
| EIA Neg n=254 | POS | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| | IND | 41 (16.1) | 0 (0.0) | 1 (0.4) | 2 (0.8) | 0 (0.0) | 1 (0.4) | 10 (3.9) | 0 (0.0) | 8 (3.1) | 2 (0.8) | 25 (9.8) |
| EIA RR[e] n=241 | POS | 22 (9.1) | 22 (9.9) | 22 (9.1) | 21 (8.7) | 18 (7.5) | 21 (8.7) | 21 (8.7) | 21 (8.7) | 21 (8.7) | 17 (7.0) | 0 (0.0) |
| | IND | 128 (53.1) | 1 (0.4) | 0 (0.0) | 6 (2.5) | 18 (7.5) | 16 (6.6) | 7 (2.9) | 2 (0.8) | 31 (12.9) | 76 (31.5) | 39 (16.2) |

| High Risk[b] | HIV-1 WB Result | | Band Specificities (# and % of samples)[d] | | | | | | | | | Non-Viral |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | gp160 | gp120 | p65 | p55 | p51 | gp41 | p31 | p24 | p18 | |
| EIA Neg n=195 | POS | 1 (0.5) | 1 (0.5) | 1 (0.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (0.5) | 0 (0.0) | 0 (0.0) |
| | IND | 41 (21.0) | 4 (2.1) | 2 (1.0) | 4 (2.1) | 2 (1.0) | 3 (1.5) | 14 (7.2) | 2 (1.0) | 2 (1.0) | 1 (0.5) | 33 (16.9) |
| EIA RR[e] n=150 | POS | 140 (93.3) | 140 (93.3) | 140 (93.3) | 136 (90.7) | 107 (71.3) | 137 (91.3) | 138 (92.0) | 133 (88.7) | 134 (89.3) | 115 (76.7) | 0 (0.0) |
| | IND | 3 (2.0) | 2 (1.3) | 0 (0.0) | 2 (1.3) | 18 (7.5) | 1 (0.7) | 1 (0.7) | 0 (0.0) | 2 (1.3) | 76 (31.5) | 1 (0.7) |

| AIDS[c] | HIV-1 WB Result | | Band Specificities (# and % of samples)[d] | | | | | | | | | Non-Viral |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | gp160 | gp120 | p65 | p55 | p51 | gp41 | p31 | p24 | p18 | |
| EIA Neg n=2 | POS | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| | IND | 2 (100.0) | 1 (50.0) | 0 (0.4) | 2 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (50.0) | 0 (0.0) | 0 (0.0) |
| EIA RR[e] n=184 | POS | 178 (96.7) | 178 (96.7) | 178 (96.7) | 167 (90.8) | 80 (43.5) | 165 (89.7) | 178 (96.7) | 156 (84.8) | 161 (87.5) | 95 (51.6) | 0 (0.0) |
| | IND | 6 (3.3) | 6 (3.3) | 5 (2.7) | 1 (0.5) | 0 (0.0) | 1 (0.5) | 2 (1.1) | 0 (0.0) | 1 (0.5) | 0 (0.0) | 0 (0.0) |

a. Persons with no known risk factors including normal blood donors.

b. Homosexuals, IDUs, and other accepted risk designations.

c CDC Classification; MMWR 1982; 31: 507-508.

d. Band patterns for negative samples do not appear in this table. By definition, negative samples show no reactivity.

e. RR indicates repeatedly reactive EIA results.

Table 2 summarizes Western blot results relative to the appearance of AIDS associated symptoms within EIA repeatedly reactive individuals. Since all samples were obtained from repositories, clinical symptoms were unknown in some cases.





## Table 2: HIV-1 Western Blot Results of EIA Repeatedly Reactive Samples Relative to Clinical Status

| | | | Western Blot (# and %) | | |
|---|---|---|---|---|---|
| | | EIA RR | Neg | Ind | Pos |
| Low Risk | Asymptomatic | 3 | 0 | 0 | 3 (100.0) |
| | Unknown | 238 | 91 (38.2) | 128 (53.8) | 19 (8.0) |
| | Total | 241 | 91 (37.8) | 128 (53.1) | 22 (9.1) |
| High Risk[1] | Asymptomatic | 25 | 0 | 1 (4.0) | 24 (96.0) |
| | Symptomatic | 186 | 0 | 6 (3.2) | 180 (96.8) |
| | Unknown | 123 | 6 (4.9) | 0 | 117 (95.1) |
| | Total | 334 | 6 (1.8) | 7 (2.1) | 321 (96.1) |

[1] Includes clinically diagnosed AIDS.

### Sensitivity and Specificity

Gold standard??

Sensitivity and specificity of the HIV-1 Western Blot Kit was determined in comparative studies with a previously licensed HIV-1 Western blot using EIA repeatedly reactive samples from high AIDS risk and low risk populations respectively. The data are summarized in Table 3.

Among 256 EIA repeatedly reactive samples from high risk groups, the HIV-1 Western Blot Kit was positive in 204/204 (100%) samples identified as positive by the previously licensed test, demonstrating equivalent sensitivity.

Among 210 EIA repeatedly reactive samples from low risk groups, the HIV-1 Western Blot Kit was negative in 38/52 (73%) of samples identified as negative by the previously licensed test and indeterminate (not falsely positive) in the remaining 14 samples. Thus, there were 0% false positive tests. These data demonstrate comparable specificity of this test.

## Table 3: Comparative Study of Western Blot Results in High and Low Risk Populations

| | | High Risk EIA RR | | | | Low Risk EIA RR | | | |
|---|---|---|---|---|---|---|---|---|---|
| | WB Result | HIV-1 Western Blot Kit | | | Licensed Kit Total | HIV-1 Western Blot Kit | | | Licensed Kit Total |
| | | Pos | Ind | Neg | | Pos | Ind | Neg | |
| *Previously Licensed kit* | *Pos* | 204 | 0 | 0 | 204 | 15 | 0 | 0 | 15 |
| | *Ind* | 40 | 8 | 2 | 50 | 1 | 105 | 37 | 143 |
| | *Neg* | 0 | 1 | 1 | 2 | 0 | 14 | 38 | 52 |
| | **Totals** | **244** | **9** | **3** | **256** | **16** | **119** | **75** | **210** |

In the comparative study on high risk samples, the overall concordance rate was 83% for positive, indeterminate and negative interpretations. The rate of indeterminate test results was 3.5% for this test and 19% for the previously licensed test.

In the comparative study on low risk samples, the overall concordance rate was 75% for positive, indeterminate and negative interpretations. The rate of indeterminate test results was comparable for this test (57%) and the previously licensed test (68%).

Neither test was positive for a sample which was identified as negative by the other test.

To eliminate any bias due to the different interpretive criteria used in the two kits, the comparative data in Table 3 is presented in Table 4 using the same criteria to interpret both tests.

**Table 4: HIV-1 Western Blot Kit and the Previously Licensed Kit Results Interpreted by HIV-1 Western Blot Kit Criteria**

| HIV-1 WB Kit Criteria: 2 of 3: gp160/gp120, p24, gp41 | | High Risk EIA RR | | | | Low Risk EIA RR | | | |
|---|---|---|---|---|---|---|---|---|---|
| | WB Result | HIV-1 Western Blot Kit | | | Licensed Kit Total | HIV-1 Western Blot Kit | | | Licensed Kit Total |
| | | Pos | Ind | Neg | | Pos | Ind | Neg | |
| *Previously Licensed kit* | *Pos* | 231 | 0 | 0 | 231 | 16 | 3 | 3 | 22 |
| | *Ind* | 13 | 8 | 2 | 23 | 0 | 102 | 34 | 136 |
| | *Neg* | 0 | 1 | 1 | 2 | 0 | 14 | 38 | 52 |
| | **Totals** | **244** | **9** | **3** | **256** | **16** | **119** | **75** | **210** |

## Precision

Reproducibility was evaluated in an in-house study. A panel of eight independent specimens was tested on each of seven separate kit lots. Seven different technicians evaluated bands and interpreted results. The percent of times each band was scored reactive is presented in Table 5 below.

These results demonstrate that for strongly reactive specimens and negative specimens, reproducibility is high. The weakly reactive specimens (ID #s 4 and 5) were less than 100% reproducible for most bands. In these specimens, final result interpretation was not affected.

**Table 5: Reproducibility of the HIV-1 WB Kit**

| Specimen | EIA OD | Cutoff | Reactivity | Percent Frequency of Bands | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | gp160 | gp120 | p65 | p55 | p51 | gp41 | p31 | p24 | p18 |
| 1 | >2.0 | 0.232 | RR | 100 | 100 | 100 | 80 | 100 | 100 | 100 | 100 | 100 |
| 2 | >2.0 | 0.232 | RR | 100 | 100 | 100 | 90 | 100 | 100 | 100 | 100 | 100 |
| 3 | >2.0 | 0.232 | RR | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 4 | 1.384 | 0.232 | RR | 100 | 90 | 10 | 100 | 10 | 60 | 10 | 100 | 0 |
| 5 | 0.431 | 0.232 | RR | 100 | 70 | 100 | 60 | 100 | 100 | 100 | 90 | 0 |
| 6 | 0.026 | 0.232 | Neg | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 0.024 | 0.232 | Neg | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | >2.0 | 0.232 | RR | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

## INTERFERING FACTORS AND SUBSTANCES

Testing was performed on specimens from individuals with clinical conditions unrelated to HIV-1 which might result in a reactivity with proteins present. Samples studied included 25 from persons with autoimmune diseases, 12 with elevated gammaglobulins, 110 with viral infections unrelated to HIV-1 and 38 other conditions. The viral infections included samples positive in clinical tests for Cytomegalovirus (12), Infectious Mononucleosis (10), Epstein-Barr virus (3), Rubella (12), Varicella-Zoster (3), Herpes Simplex (12), HBsAg (7), and HTLV-I (39). Although bands were occasionally present at viral locations, none of the strips could be interpreted as positive.





Studies were also performed on 34 samples possessing reactivity to 19 different HLA specificities. Bands were present on nine samples, but only at non-viral locations. Results are presented in Table 6 below.

Further studies were performed on serum and plasma collected in a variety of anticoagulants. Data indicate no interference in interpretation from specimen exposure to anticoagulants. All specimens known to be HIV-1-Positive were interpreted as WB-Positive; all specimens known to be HIV-1-Negative were interpreted as WB-Negative. ***No non-viral*** bands were observed.

**Table 6: Results of Samples Representing Disease Processes Other than HIV-1 Infection and Other Interfering Factors**

| Result | n= | Band Distribution (Number and %) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | gp160 | gp120 | p65 | p55 | p51 | gp41 | p31 | p24 | p18 | Non-viral |
| Neg | 127<br>61.4% | 0<br>0.0% | 0<br>0.0% | 0<br>0.0% | 0<br>0.0% | 0<br>0.0% | 0<br>0.0% | 0<br>0.0% | 0<br>0.0% | 0<br>0.0% | 0<br>0.0% |
| Ind | 80<br>38.6% | 0<br>0.0% | 0<br>0.0% | 2<br>2.5% | 6<br>7.5% | 3<br>3.8% | 2<br>2.5% | 0<br>0.0% | 2<br>2.5% | 4<br>5.0% | 73<br>91.2% |
| TOTAL | 207<br>100% | 0<br>0.0% | 0<br>0.0% | 2<br>1.0% | 6<br>2.9% | 3<br>1.4% | 2<br>1.0% | 0<br>0.0% | 2<br>1.0% | 4<br>2.0% | 73<br>35.3% |

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# ATTACHMENT 4

# HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1)

OraSure®

## HIV-1 Western Blot Kit

An Enzyme Immunoassay for the Detection of Antibodies to Human Immunodeficiency Virus Type 1 (HIV-1) in Human Oral Fluid Specimens Obtained with OraSure® HIV-1 Oral Specimen Collection Devices

20 Tests

Store at 2-8˚C

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## NAME AND INTENDED USE

The OraSure® HIV-1 Western Blot Kit is an *in vitro* qualitative assay for the detection of antibodies to individual proteins of the Human Immunodeficiency Virus Type 1 (HIV-1) in human oral fluid specimens obtained with the OraSure HIV-1 Oral Specimen Collection Device. For convenience, the collection device will hereafter be referred to as "OraSure". The OraSure HIV-1 Western Blot Kit is intended for use as an additional, more specific test for HIV-1 antibodies in OraSure specimens collected from individuals of unknown risk for HIV-1, which are found to be repeatedly reactive by the Oral Fluid Vironostika® HIV-1 Microelisa System. The OraSure HIV-1 Western Blot Kit is intended for professional use only.

The OraSure HIV-1 Western Blot Kit is not intended for use with blood, serum/plasma or urine specimens or for screening potential blood donors.

## SUMMARY AND EXPLANATION OF THE TEST

Acquired Immunodeficiency Syndrome (AIDS) is caused by at least two etiologic agents which are designated as Human Immunodeficiency Virus Type 1 (HIV-1) and Human Immunodeficiency Virus Type 2 (HIV-2).[1,2] Infections with HIV-2 are found primarily in parts of West Africa.[2] Current data indicate that HIV infections are transmitted by sexual contact, exposure to blood (including sharing needles and syringes) or certain blood products, and perinatally by mother to infant.[3,4] Published data have established that patients with AIDS and individuals infected with HIV-1 produce antibodies against HIV-1 proteins. Studies have shown that HIV-1 antibodies have been detected in saliva samples of most HIV-infected patients.[5-7]

A number of EIA kits are currently available for the screening of serum specimens for HIV-1 antibodies. The Oral Fluid Vironostika HIV-1 Microelisa System manufactured by bioMérieux, Inc. is available specifically for screening OraSure oral fluid specimens. These specimens are obtained using the OraSure HIV-1 Oral Specimen Collection Device which is designed to collect oral HIV-1 antibodies while minimizing problems inherent in saliva samples (namely in high viscosity and instability). Samples found to be repeatedly reactive for HIV-1 antibodies are tested using additional, more specific tests, such as Western blot or immunofluorescence assays. The Western blot assay, as described by Tsang et al,[8] is useful for elucidating the specificity of the antibody response to HIV-1 (a summary of the principles of the assay is presented on page 2).

Clinical serum samples that are reactive in the screening assays but do not contain HIV-1 antibodies have also been described.[9] Some of these samples possess antibodies to certain Class II HLA histocompatibility antigens that are found in some cell lines used to produce the virus. Other individuals, who have had no known exposure to HIV-1, produce reactive results in the screening test for unknown reasons. Such nonspecific results are found commonly when screening tests are used in low risk populations. Since the psychosocial and medical implications of a positive antibody test may be significant, it is recommended that additional testing be performed on such samples to validate the presence of antibodies specific to HIV-1.[10]

The OraSure HIV-1 Western Blot Kit was developed in order to provide an additional, more specific assay for HIV-1 antibody detection in OraSure specimens found to be repeatedly reactive in the screening EIA. The OraSure HIV-1 Western

Blot Kit, when used as directed in this insert, will detect antibodies to HIV-1 when present in human oral fluid samples obtained with the OraSure HIV-1 Oral Specimen Collection Device. The position of bands on the preblotted nitrocellulose strips allows the antibody reactivity to be associated with specific viral antigens. An OraSure sample that is reactive in both EIA screening test and Western blot assay is presumed to be positive for antibodies to HIV-1, indicating infection with this virus except in situations of passively acquired antibody or experimental vaccination. Antibodies to HIV-2 may also react with the protein antigens of HIV-1.[11] Therefore, individuals infected with HIV-2 may have reactive tests in the HIV-1 Western blot assay. Usually, however, the cross-reactivity is incomplete, resulting in an indeterminate test result (see Interpretation of Results section). Absence of antibodies to HIV cannot be taken as absolute proof that an individual is free of HIV-1 or incapable of transmitting the virus. Individuals with positive tests should be referred for medical evaluation.

## CHEMICAL AND BIOLOGICAL PRINCIPLES OF THE PROCEDURE

The whole cell viral lysate used in the manufacture of the OraSure HIV-1 Western Blot Kit is manufactured by bioMérieux, Inc. (U.S. license number 956). It is HIV-1 propagated in an H-9/HTLV-IIIB, T-lymphocyte cell line. It is purified by ultra-centrifugation and inactivated by treatment with nonionic detergent and heat.

When used to manufacture the preblotted strips, inactivated and denatured proteins of the HIV-1 virus are fractionated by SDS-polyacrylamide gel electrophoresis. The resolved protein bands are electrophoretically transferred to nitrocellulose sheets. These preblotted nitrocellulose sheets are cut into strips.

OraSure HIV-1 specimens, diluted in Sample Buffer, are incubated with the preblotted nitrocellulose strips. If antibodies to specific HIV-1 proteins are present in a specimen, they bind to epitopes contained in the proteins banded on the strip. Any antibody not bound is removed by washing. The conjugate, alkaline phosphatase-labeled goat anti-human immunoglobulin, is then added to the strip and allowed to incubate. It binds to antibodies already bound to viral proteins on the strip. Excess conjugate is removed by washing. The strips are then incubated with a substrate specific to the alkaline phosphatase. The color reaction is stopped by aspiration and washing.

If antibodies to specific HIV-1 proteins (p) or glycoproteins (gp) are present in the specimen in sufficient concentration, purple bands may be visible at one or more of the following positions on the nitrocellulose strip: gp160, gp120, p65, p55, p51, gp41, p31, p24, and p18 (number refers to apparent molecular weight in kilodaltons).

## KIT COMPONENTS SUPPLIED

(20 Test Kit)

**OraSure® HIV-1 Western Blot Strips** **20 strips**

Prenumbered nitrocellulose strips, preblotted with resolved HIV-1 proteins; packed in a resealable plastic pouch between buffer-soaked absorbent paper; buffer contains 0.1% sodium azide as a preservative.

**Substrate** **1 bottle; 22 mL**

BCIP/NBT single reagent substrate in an organic base/TRIS buffer.

**Powdered Milk** **1 bottle; 30 g**

Non-fat milk solids.

**Sample Diluent Concentrate** **1 bottle; 100 mL**

Phosphate buffered saline with 3.0% Tween-20; contains 0.01% thimerosal as a preservative.

**Conjugate Concentrate** **1 vial; 0.25 mL**

Goat anti-human IgG (heavy and light chains) $F(ab')_2$ fragment, labeled with alkaline phosphatase; contains 0.1% sodium azide as a preservative.

**OraSure® HIV-1 WB Negative Control** **1 vial; 0.65 mL**

Human serum or plasma, nonreactive for antibodies to HIV-1, in OraSure Control Matrix; tested negative for HBsAg and antibodies to HCV; contains a proprietary preservative.

**OraSure® HIV-1 WB Low Positive Control** **1 vial; 0.65 mL**

Human serum or plasma, reactive for antibodies to HIV-1, in OraSure Control Matrix; tested negative for HBsAg and antibodies to HCV; heat-inactivated to render material noninfectious for HIV-1; contains a proprietary preservative.

**OraSure® HIV-1 WB High Positive Control** **1 vial; 0.65 mL**

Human serum or plasma, reactive for antibodies to HIV-1, in OraSure Control Matrix; tested negative for HBsAg and antibodies to HCV; heat-inactivated to render material noninfectious for HIV-1; contains a proprietary preservative.

**Reaction Trays** **5 each**

Eight lane disposable trays with lids.

**Caution:** The Negative Control is prepared from human plasma or serum found to be nonreactive for HIV-1 antibodies and tested for Hepatitis B surface antigen (HBsAg) and antibodies to HCV by FDA-licensed methods. The Positive Controls are prepared from anti-HIV-1 positive human plasma or serum, which was heat-inactivated to render it noninfectious for HIV-1. However, as no procedure can offer complete assurance that infectious agents are absent, all specimens of human origin should be considered potentially infectious and handled with care.[12]

## EQUIPMENT REQUIRED BUT NOT SUPPLIED

1. Centrifuge tube for each sample to be processed
2. Centrifuge
3. Refrigerator (2-8˚C)
4. 37˚C water bath
5. Graduated cylinders
6. Beaker or appropriate mixing vessel
7. Balance
8. Laboratory timer
9. Magnetic stir plate and stir bar
10. Test Tubes: glass, polypropylene, or polystyrene (12x75 mm) (optional)
11. Precision micropipets to deliver variable volumes from 5 to 1000 µL
12. Disposable pipet tips
13. Graduated pipets to deliver volumes to 25 mL
14. Scissors
15. Forceps for strip handling (plastic or Teflon-coated)
16. Transfer pipets
17. Rotary platform, capable of rotating at 50-60 rpm
18. Aspiration system
19. Repeating pipet to deliver 1-2 mL volumes

## KIT STORAGE AND STABILITY

1. Store all components at 2-8˚C when not in use.
2. Expiration dates printed on the kit and kit components indicate the limits of stability.
3. Stability of the components after reconstitution or dilution is as follows:
   a. Sample Buffer
      Store at room temperature while performing assay. Discard excess buffer at completion of assay.
   b. Sample dilutions
      Test tube sample dilutions must be applied to the strips within one hour of dilution. The dilutions are stored at room temperature during that time.
   c. Conjugate dilution
      Conjugate dilution must be prepared during the last five minute wash and applied immediately after the wash is complete and the wash solution is aspirated. Excess Conjugate dilution must be discarded.
   d. All other kit components are supplied ready to use.
4. Keep strip pouch tightly sealed. *Do not let strips dry out.*

## CHEMICAL OR PHYSICAL INDICATIONS OF INSTABILITY

Alterations in physical appearance of kit materials may indicate instability or deterioration.

**Note**: Sample Diluent Concentrate may contain crystals. This will not affect assay performance if crystals are dissolved before use (see page 8, step 1.a).

Substrate is pale yellow in color. A fine black precipitate may be observed but its presence does not affect product performance.

## SPECIMEN COLLECTION, STORAGE AND PREPARATION

**Note**: This test kit may not be used to assay blood specimens. This test kit may only be used to assay OraSure HIV-1 oral fluid specimens obtained using the OraSure HIV-1 Oral Specimen Collection Device.

A. Specimen Collection
   1. Refer to the OraSure HIV-1 Oral Specimen Collection Device package insert for instructions on collecting a specimen.
   2. OraSure HIV-1 specimens must be transported to the laboratory in the OraSure HIV-1 Specimen Vial.
   3. OraSure HIV-1 specimens may be transported to the laboratory at ambient temperature via courier, air freight, or regular mail. OraSure specimens should be protected from impact, direct sunlight, and temperatures exceeding 37˚C (98˚F). Federal, state and local regulations regarding transportation of diagnostic specimens are applicable to OraSure HIV-1 specimens.

B. Specimen Storage
   1. After receipt at the laboratory, OraSure HIV-1 specimens (on or off the collection pad) should be stored at 2-8˚C. Specimens may be stored at 4˚C to 37˚C for a maximum of 21 days from the time of collection, including the time for shipping and testing. If testing of specimens cannot be completed within 21 days, OraSure HIV-1 specimens can be stored frozen at -20˚C for a maximum of six weeks.
   2. OraSure HIV-1 specimens frozen and thawed once must be tested within the 21 days (see B.1 above). Specimens frozen and thawed twice must be tested within 24 hours, or discarded.

C. Specimen Preparation
   1. Record the specimen identification number from the OraSure HIV-1 Specimen Vial.
   2. Ensure that the specimen is within acceptable dating for testing, i.e., <21 days from collection. Note: All testing should be completed within 21 days of specimen collection unless stored at -20˚C (see B.1 above).
   3. Hold the vial with the pointed tip up.
   4. Move the pad away from the vial tip by gently tapping the vial.
   5. Break the pointed tip of the vial off with the thumb.
   6. Place a centrifuge tube over the vial and invert the tube and vial.
   7. Centrifuge at 600-800 x g force for 15 minutes.
   8. Determine that there is a minimum of 0.75 mL volume of specimen eluate.

If the volume of the centrifuged specimen is less than 0.75 mL, the specimen is unsuitable for testing and a new specimen from the test subject must be obtained. Notify the ordering physician if the volume of specimen is insufficient.

## PRELIMINARY PRECAUTIONS

1. Keep testing area separate from areas where blood or blood products for transfusion are stored.
2. Do not pipet by mouth.
3. Do not smoke, eat, or drink while handling test materials.
4. Wear disposable gloves throughout the specimen processing and testing procedure.
5. Handle all materials used in the test (including specimens, Sample Buffer, reaction trays and pipets) as though capable of transmitting infectious agents.[12]
6. Consult a physician immediately in the event that contaminated materials are ingested or come in contact with mucous membranes or breaks in the skin.
7. Immediately clean up any spills containing potentially infectious material with freshly prepared 1:10 dilution of ≥5% sodium hypochlorite (bleach) and dispose of the cleaning material by an appropriate method.
8. Dispose of all specimens and materials used to perform the test as if they contain infectious agents. Prior to disposal, treat as follows:

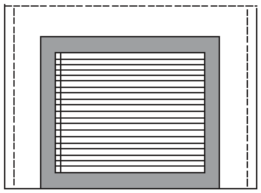
| Material | Disposal Procedure |
|---|---|
| Reusable items | Autoclave for 60 minutes at 121˚C. |
| Disposable items | Incinerate. |
| Liquid waste | Mix with bleach to yield a final ratio of one part bleach to nine parts waste (1:10). Allow the mixture to stand 30 minutes before flushing down the drain. |

## PROCEDURAL NOTES AND PRECAUTIONS

**Note**: This test kit may only be used to assay OraSure HIV-1 oral fluid specimens obtained using the OraSure HIV-1 Oral Specimen Collection Device. Do not test any specimens other than OraSure oral fluid specimens with this kit.

1. Do not interchange or combine any kit component, including strips, with components of another kit lot.
2. The OraSure Low Positive and Negative Controls must be assayed with ***each*** run. The OraSure High Positive must be assayed with the first run of every package of strips, but is optional in subsequent runs. This High Positive Control strip should be retained as a reference. It will be compared to the test strips run from that package to determine band identification and placement.
3. Do not perform the test in the presence of reactive vapors (e.g., from acids, alkalis, or aldehydes), dust, or residual bleach or bleach fumes; the enzymatic activity of the conjugate may be affected or reactivity may be decreased.
4. Prepare an assay worksheet, ensuring that the patient sample and control identification is linked to the number embossed on the nitrocellulose strip.
5. For sample dilution and addition to nitrocellulose strips, two options are offered (explained in detail on pages 8 and 9).
   a. The first involves preparing the dilutions in test tubes and adding them to the strips.
   b. The second involves adding the sample directly into the trough which contains the strip and Sample Buffer.

6. Avoid contamination of the strips and/or the buffer-soaked absorbent paper in the resealable pouch during handling (this may cause false reactivity in subsequent assays).
   a. Prior to removing the strips from the pouch, clean the work surface and forceps with isopropyl alcohol.
   b. Change gloves prior to opening the pouch.
   c. Always use clean forceps when handling strips.
   d. For initial use of strips, cut pouch below the seal line, keeping upper portion of pouch intact including seal line.
   e. It is recommended that the lower portion of the strip pouch be cut on the remaining two sides (dotted lines as shown in diagram to the right).
   f. Fold the plastic down, which acts as a protective barrier, to expose the strips.



7. Place each prenumbered strip, with the green indicator line facing up, in the reaction trays in numerical order. This facilitates band alignment, for ease of reading results.
8. Do not allow strips to dry out prior to sample addition. If diluting the samples in test tubes, place strips into trays only *after* dilutions have been made.
9. As soon as the sample dilutions have been added to all strips in a tray, cover the tray with a lid.
10. **It is essential to avoid cross-contamination between troughs, especially prior to and during sample incubation.**
    a. Add sample to the trough of the reaction tray, using a transfer pipet for diluted samples or a pipet for undiluted samples.
    b. It is suggested that an additional precaution be taken by positioning the strips in every other trough of a tray.
    c. Avoid delivering bubbles to the liquid in the troughs.
    d. Be careful to avoid dislodging fluid from the troughs when transferring trays.
    e. Liquid in the troughs should not contact tray lids (if liquid should contact tray lids, immediately remove the material with a lab wipe).
11. It is important that the items used to prepare and dispense Sample Buffer be scrupulously clean (a repeating pipet is preferable for dispensing the 1-2 mL of Sample Buffer).
12. Prime the pipet tip when measuring samples or reagents.
13. A rotation speed of 50-60 rpm is recommended for each rotation step.
14. Be certain that each strip is immersed in the liquid and moves freely; however, liquid must not contact the tray lid during rotation.
15. Incomplete or ineffective washing will compromise the assay; it is imperative to follow the wash procedure carefully.
16. Discard used disposable reaction trays as biohazardous waste. Reuse of the trays and lids is <u>not</u> recommended.
17. Samples must be at room temperature (20-25˚C) before starting the test.

18. Reagents should be at room temperature (20-25˚C) before beginning the assay except for Conjugate Concentrate and Substrate, which must both remain refrigerated (2-8˚C) until just prior to use. Return all reagents to 2-8˚C after use.

**CAUTION:** The Conjugate Concentrate and the buffer in the absorbent paper surrounding the strips contain sodium azide. If discarding into the sewer system, flush copiously with water. This helps prevent formation of metallic azides which, when highly concentrated in metal plumbing, may be potentially explosive. Decontaminate plumbing according to CDC guidelines.[13]

## ORASURE HIV-1 WESTERN BLOT TEST PROCEDURE

1. Prepare Sample Buffer as follows:
   a. Check Sample Diluent Concentrate for crystals.
      i. If crystals have formed, dissolve them by warming the entire bottle and its contents in a 37˚C water bath for 10 minutes or until crystals are completely dissolved.
      ii. Allow the material to reach room temperature before use.
   b. Determine the volume of Sample Buffer to be prepared and quantity of each constituent required from the chart below.

| Total number of strips to be assayed† | mL of Sample Buffer to prepare* | mL of Deionized $H_2O$ required** | mL of Sample Diluent Conc. required** | g of Powdered Milk required*** |
|---|---|---|---|---|
| 3 - 6 | 200 | 180 | 20 | 6 |
| 7 - 9 | 300 | 270 | 30 | 9 |
| 10 - 13 | 400 | 360 | 40 | 12 |
| 14 - 16 | 500 | 450 | 50 | 15 |
| 17 - 20 | 600 | 540 | 60 | 18 |

† Include strips for controls.
* 30.0 mL of Sample Buffer is required for each strip assayed.
** Sample Diluent Concentrate is diluted 1:10 in deionized water.
*** 3 g of Powdered Milk is required for every 100 mL of diluted Sample Diluent Concentrate.

   c. Combine the required amounts of deionized water, Sample Diluent Concentrate and Powdered Milk.
   d. Mix the solution for a minimum of 15 minutes (ensure the Powdered Milk is completely dissolved).
   e. Store at room temperature while performing assay. Discard excess buffer at completion of assay.
2. Prepare an assay worksheet, ensuring that the patient sample and control identification is linked to the number embossed on the nitrocellulose strip.
3. Both the OraSure Low Positive and Negative Controls must be assayed with ***each*** run. The OraSure High Positive must be assayed with the first run of every package of strips, but is optional in subsequent runs. This High Positive Control strip should be retained as a reference. It will be compared to the test strips run from that package to determine band identification and placement.
4. Add controls and patient specimens to nitrocellulose strips by one of the following methods:

a. **METHOD I: Test Tube Dilution**

i. Into appropriately labeled test tubes, add 150 µL of each specimen or control to 1.0 mL of Sample Buffer and mix well. These dilutions must be tested within an hour.

ii. Place one prenumbered strip with *green indicator line facing up* into each trough as follows, ensuring the strips do not dry out:

a) For initial use of strips, cut pouch below the seal line, keeping upper portion of pouch intact including seal line.

b) Cut the lower portion of the pouch on the two remaining sides (see page 7, step 6.e).

c) Fold the packaging back to expose strips.

d) Beginning with the left side of the series (strip #1), remove strips to be assayed and place them in numerical order.

e) Grasp the strip at the green indicator line with forceps.

f) Transfer the strips, avoiding contact with contaminated surfaces, into the troughs of the reaction tray(s).

g) Place any remaining strips (still encased in moist blotting paper and contained in the lower portion of the pouch) in the upper portion of pouch, seal using zip closure, and return to storage at 2-8˚C.

iii. Transfer the contents of each tube (~1.1 mL) into the corresponding trough using a transfer pipet.

b. **METHOD II: On-Strip Dilution**

i. Add 1.0 mL Sample Buffer to each trough to be used.

ii. Place one prenumbered strip with *green indicator line facing up* into each trough as follows, ensuring the strips do not dry out:

a) For initial use of strips, cut pouch below the seal line, keeping upper portion of pouch intact including seal line.

b) Cut the lower portion of the pouch on the two remaining sides (see page 7, step 6.e).

c) Fold the packaging back to expose strips.

d) Beginning with the left side of the series (strip #1), remove strips to be assayed and place them in numerical order.

e) Grasp the strip at the green indicator line with forceps.

f) Transfer the strips, avoiding contact with contaminated surfaces, into the troughs of the reaction tray(s).

g) Place any remaining strips (still encased in moist blotting paper and contained in the lower portion of the pouch) in the upper portion of pouch, seal using zip closure, and return to storage at 2-8˚C.

iii. Add 150 µL of each OraSure specimen or control to the corresponding trough.

5. Cover each tray with a lid and mix by gentle rotation (50-60 rpm) on a rotator for 180 minutes (3 hours) at room temperature.

6. After incubation, completely aspirate the liquid from troughs (do not allow the strips to dry).

7. Wash the strips as follows:
   a. Add 2.0 mL Sample Buffer to each strip.
   b. Immediately aspirate all liquid from each trough.
   c. Repeat steps a and b two more times.
   d. Add 2.0 mL Sample Buffer to each strip and replace the lid(s).
   e. Place the tray(s) on the rotator (at 50-60 rpm) for 5 minutes.
   f. Aspirate the Sample Buffer completely.
   g. Repeat steps d and e one more time.
8. Prepare diluted Conjugate during the final wash step (step 7.g above):
   a. Conjugate dilution must be prepared during the last five minute wash and applied immediately after the wash is complete and the wash solution is aspirated. Excess Conjugate dilution must be discarded.
   b. Remove Conjugate Concentrate from the refrigerator.
   c. Determine volumes required of Sample Buffer and Conjugate Concentrate from the following chart:

| Total number of strips being assayed† | mL of Sample Buffer required* | µL of Conjugate Concentrate required** | Total number of strips being assayed† | mL of Sample Buffer required* | µL of Conjugate Concentrate required** |
|---|---|---|---|---|---|
| 3 | 8 | 40 | 12 | 26 | 130 |
| 4 | 10 | 50 | 13 | 28 | 140 |
| 5 | 12 | 60 | 14 | 30 | 150 |
| 6 | 14 | 70 | 15 | 32 | 160 |
| 7 | 16 | 80 | 16 | 34 | 170 |
| 8 | 18 | 90 | 17 | 36 | 180 |
| 9 | 20 | 100 | 18 | 38 | 190 |
| 10 | 22 | 110 | 19 | 40 | 200 |
| 11 | 24 | 120 | 20 | 42 | 210 |

† Include strips for controls.
* Prepare 2.0 mL of diluted Conjugate for each strip assayed (a small excess has been incorporated for pipetting ease).
** Conjugate Concentrate is diluted 1:201.

   d. Prepare Conjugate dilution by combining required amounts of Conjugate Concentrate and Sample Buffer, and mix well.
   e. Return the remaining Conjugate Concentrate to storage at 2-8°C.
9. Aspirate the Sample Buffer from the troughs and add 2.0 mL diluted Conjugate to each strip.
10. Replace each lid and incubate on the rotator (at 50-60 rpm) for 45 minutes at room temperature.
11. Repeat wash procedure from steps 6 and 7.
12. Completely aspirate the Sample Buffer and add 2.0 mL of deionized water to each strip.
13. Replace each lid and place on rotator (at 50-60 rpm) for 5 minutes.
14. Remove Substrate from refrigerator during final wash (step 13).
15. Aspirate deionized water from each strip.
16. Add 1.0 mL of Substrate to each strip.

17. Replace each lid and *gently* move trays back and forth 2-3 times by hand on work surface to ensure strips are completely immersed in Substrate.
18. Incubate for exactly 10 minutes at room temperature without rotation. **Note**: It is important not to exceed the 10 minute Substrate incubation time.
19. Stop the color development of the strips as follows:
    a. Aspirate the Substrate.
    b. Add 2.0 mL of deionized water to each strip.
    c. Immediately aspirate the contents of the tray(s).
    d. Repeat steps b and c two more times.
    e. Add 2.0 mL deionized water to each strip and replace the tray lid(s).
    f. Place on rotator for 5 minutes.
    g. Aspirate water completely.
20. Allow the developed strips to air dry in the tray(s).
21. Handle the strips carefully; use clean forceps to remove from troughs.
22. Read and interpret the **dry** strips as soon as possible, since developed strips exposed to light may experience fading of bands. Store developed strips in the dark at room temperature.

## QUALITY CONTROL

Both the OraSure Low Positive and Negative Controls must be assayed regardless of the number of samples tested. The OraSure High Positive must be assayed with the first run of every package of strips, but is optional in subsequent runs. This High Positive Control strip should be retained as a reference. It will be compared to the test strips run from that package to determine band identification and placement.

The following conditions must be met for the assay to be considered valid:

1. Negative Control: No bands are observed on the strip.
2. Low Positive Control (*Weakly Reactive*): Bands are present (P) at gp160, gp41, and p24. Other bands may or may not be visible.
3. High Positive Control (*Strongly Reactive*): Bands are present (P) at gp160, gp41 and p24. Bands are visible at gp120, p65, p51, p31, and p18. The p55 band may or may not be visible (see Figure 1, page 13).

## INTERPRETATION OF RESULTS

1. Band Identification
   a. Correlate the band position of the OraSure High Positive Control strip with Figure 1 on page 13 to identify the HIV-1 viral bands and their positions.
   b. Compare each test strip to the OraSure High Positive Control strip for identification of reactive bands.
2. Band Intensity
   a. Compare the bands of each strip and control to the gp41 band on the OraSure Low Positive Control strip and assign a level of intensity as follows:

| **Assignment** | **Definition** |
|---|---|
| Present (P) | The band intensity is greater than or equal to the gp41 band on the OraSure Low Positive Control strip. |
| Indeterminate (I) | The band is visible but intensity is less than the gp41 band on the OraSure Low Positive Control strip. |
| Absent (A) | No reactivity is observed. |

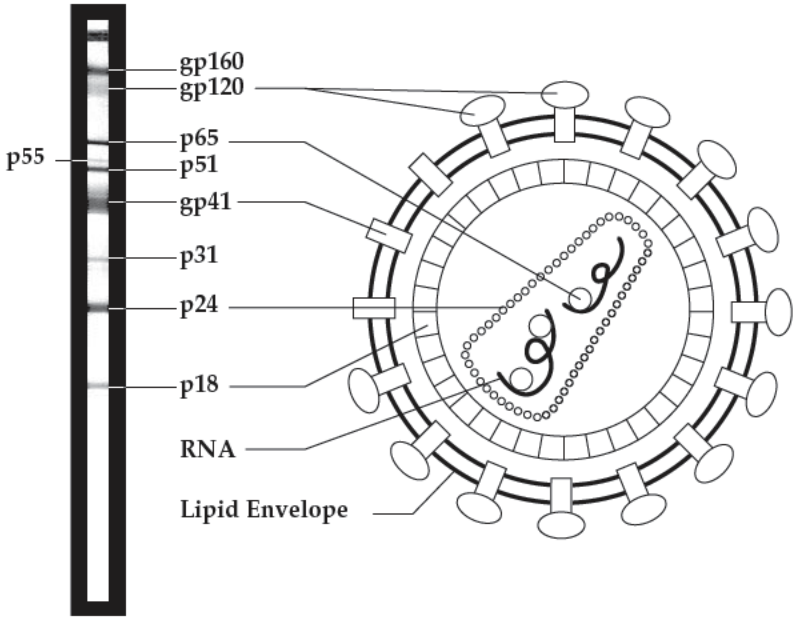
3. Strip Interpretation
   a. Based on band position and reactivity, analyze the results and assign each strip a final result.

| **Test Result** | **Definition** |
|---|---|
| Positive | Any two of the three major bands of diagnostic significance below must be *Present*.<br>gp160 and/or gp120 gp41 p24<br>Other bands may or may not be present. |
| Indeterminate | Any visible band reactivity which does not meet the criteria for a Positive result as described above. |
| Negative | Band reactivity is *Absent*. |

**Figure 1: Protein Band Identification on an OraSure® HIV-1 Western Blot Strip**

On the left is a representation of an OraSure Western blot strip developed with OraSure High Positive Control. The illustration is a reference for band identification and position (see Interpretation of Results, page 12, step 1).

On the right is a representation of the HIV-1 virus. The bands correlate to corresponding viral subpart origin.




*Actual Size*

*Figure 1. HIV-1 Virion*

**Viral Origin of HIV-1 Associated Bands**

| Virus Gene | Gene Product and Description | |
|---|---|---|
| *env* | gp160 | env protein precursor |
| | gp120 | outer env protein |
| | gp41 | transmembrane protein |
| *pol* | p65 | reverse transcriptase |
| | p51 | reverse transcriptase |
| | p31 | endonuclease |
| *gag* | p55 | core protein precursor |
| | p24 | core |
| | p18 | core |

## LIMITATIONS OF THE PROCEDURE

1. The assay must be performed in strict accordance with these instructions to obtain accurate, reproducible results.
2. Although a Positive result may indicate infection with the HIV-1 virus, a diagnosis of Acquired Immunodeficiency Syndrome (AIDS) can be made only if an individual meets the case definition of AIDS established by the Centers for Disease Control.[10] A repeat test on an independent sample should be considered to control for sample mix-up or operator error, and to verify a positive test result.
3. Individuals may present incomplete banding patterns due to the natural history of AIDS or other immunodeficiency states, e.g.:
   a. AIDS patients may lose antibody reactions to p24 and p31;
   b. Infants born to HIV-1 infected mothers, but who are uninfected may display incomplete patterns as passively acquired maternal antibodies begin to disappear;
   c. Individuals who have recently seroconverted may display incomplete band patterns;
   d. Infected patients with malignancies and individuals receiving immunosuppressive drugs may fail to develop a Positive result;
   e. Individuals infected with HTLV-I/II or HIV-2, may exhibit cross-reactivity;
   f. Individuals may develop incomplete patterns that reflect the composition of experimental HIV sub-unit vaccines that they may have received.
4. A person who has antibodies to HIV-1 is presumed to be infected with the virus, except that a person who has participated in an HIV vaccine study may develop antibodies to the vaccine and may or may not be infected with HIV. Clinical correlation is indicated with appropriate counseling, medical evaluation and possibly additional testing to decide whether a diagnosis of HIV infection is accurate.
5. Since reactivity of any degree with any of the proteins present on the strip results in an indeterminate result, all samples interpreted as Indeterminate should be repeated using the original specimen. In addition, individuals with indeterminate results should be followed for up to six months.[14]
6. Do not use this kit as the sole basis of diagnosis of HIV-1 infection.
7. A Negative result does not exclude the possibility of HIV-1 infection.
8. The OraSure HIV-1 Western Blot Kit is a biological product which, although highly consistent, does display variation from lot to lot. Examples of these variations include bands which have a slightly wavy or slanted appearance, small artifacts within the banding area, and a light smearing pattern across a set of strips. These are considered normal assay variations which infrequently affect assay interpretation. However, if they do interfere with the assay interpretation, call the assay invalid and repeat.

## PERFORMANCE CHARACTERISTICS

The performance of the OraSure HIV-1 Western Blot Kit was evaluated by comparison of OraSure results with those obtained from matched serum specimens tested by a licensed HIV-1 Western blot. These specimens were collected prospectively in a clinical study of low risk (n = 2,382), high risk (n = 698), and AIDS (n = 242) populations. In addition, non-specificity specimens (n = 248) were obtained from subjects with non-HIV-1 related medical conditions that might result in antibodies cross-reactive with HIV-1 proteins. All of the high risk and AIDS subjects, and 495 of the low risk subjects were tested by Western blot irrespective of their EIA results. EIA testing of an additional 1,887 “screen only” low risk subjects was carried out using the bioMérieux Oral Fluid Vironostika HIV-1 Microelisa System in an effort to find EIA repeatedly reactive samples (from uninfected individuals) with which to challenge the OraSure Western blot. Testing of the 1,887 “screen only” subjects identified 14 OraSure specimens as repeatedly reactive.

These 14 OraSure specimens and their matching sera were also advanced to Western blot testing. Thus, a total of 1,697 matched OraSure and serum specimens were tested by Western blot at five testing laboratories throughout the United States.

Low risk subjects, primarily normal blood donors, were persons with no known risk factors. Of the 698 high risk specimens, 363 were from homosexuals, 116 from injection drug users (IDUs), 83 from persons with multiple heterosexual contacts, and 44 from hemophiliacs. The remaining 92 high risk subjects included bisexuals, prostitutes, and individuals with other acknowledged risk factors. Specimens from 242 persons with clinically diagnosed AIDS were also tested.

The frequency of virus-specific bands and interpretation by risk group using the OraSure HIV-1 Western Blot Kit are presented in Table 1.

**Table 1. Frequency of Virus-Specific Bands ("Present" or "Indeterminate") and Interpretation of Specimens Tested by the OraSure HIV-1 Western Blot Kit**

| Low Risk[a] | OraSure HIV-1 WB Result | Band Specificities (# and % of samples)[g] | | | | | | | | | Non-Viral |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | gp160 | gp120 | p65 | p55 | p51 | gp41 | p31 | p24 | p18 | |
| EIA neg. | POS 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| n=495 | IND 100[b] (20.2) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 5 (1.0) | 0 (0.0) | 13 (2.6) | 2 (0.4) | 87 (17.6) |
| EIA RR[h] | POS 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| n=14 | IND 3[c] (21.4) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (7.1) | 0 (0.0) | 2 (14.3) |

| High Risk[d] | OraSure HIV-1 WB Result | Band Specificities (# and % of samples)[g] | | | | | | | | | Non-Viral |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | gp160 | gp120 | p65 | p55 | p51 | gp41 | p31 | p24 | p18 | |
| EIA neg. | POS 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| n=264 | IND 45[e] (17.0) | 4 (1.5) | 0 (0.0) | 6 (2.3) | 0 (0.0) | 5 (1.9) | 0 (0.0) | 0 (0.0) | 8 (3.0) | 0 (0.0) | 33 (12.5) |
| EIA RR[h] | POS 429 (98.8) | 429 (98.8) | 427 (98.4) | 417 (96.1) | 160 (36.9) | 412 (94.9) | 427 (98.4) | 394 (90.8) | 410 (94.5) | 238 (54.8) | 1 (0.2) |
| n=434[i] | IND 3 (0.7) | 3 (0.7) | 2 (0.5) | 2 (0.5) | 0 (0.0) | 2 (0.5) | 1 (0.2) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (0.2) |

| AIDS[f] | OraSure HIV-1 WB Result | Band Specificities (# and % of samples)[g] | | | | | | | | | Non-Viral |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | gp160 | gp120 | p65 | p55 | p51 | gp41 | p31 | p24 | p18 | |
| EIA neg. | POS 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| n=1 | IND 1 (100.0) | 1 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (100.0) | 0 (0.0) | 0 (0.0) |
| EIA RR[h] | POS 236 (97.9) | 236 (97.9) | 236 (97.9) | 211 (87.6) | 35 (14.5) | 207 (85.9) | 236 (97.9) | 190 (78.8) | 189 (78.4) | 87 (36.1) | 0 (0.0) |
| n=241 | IND 5 (2.1) | 5 (2.1) | 4 (1.7) | 1 (0.4) | 0 (0.0) | 1 (0.4) | 4 (1.7) | 1 (0.4) | 0 (0.0) | 1 (0.4) | 0 (0.0) |

a. Persons with no known risk factors; primarily normal blood donors.
b. 80 of 100 results Indeterminate due to non-viral bands only.
c. 2 of 3 results Indeterminate due to non-viral bands only.
d. Homosexuals, IDUs, and other accepted risk designations.
e. 32 of 45 results Indeterminate due to non-viral bands only.
f. CDC Classification; MMWR 1982; 31: 507-508.
g. Band patterns for negative samples do not appear in this table. By definition, negative samples show no reactivity.
h. RR indicates repeatedly reactive OraSure EIA results.
i. Includes two EIA false positive individuals whose OraSure Western blots were negative.

### Sensitivity Studies

The performance of the OraSure Western blot in seropositive subjects was evaluated by comparing results to those obtained by testing matched serum samples collected from individuals at high risk for HIV infection and from clinically diagnosed AIDS patients. A comparison of OraSure and serum results is presented in Table 2.

**Table 2. OraSure and Serum Western Blot Results for Confirmed Positive High Risk and AIDS Populations**

| Risk Category | Confirmed Positives | Orasure Specimen Results | | | | Serum Results | | |
|---|---|---|---|---|---|---|---|---|
| | | EIA | Western Blot | | | Western Blot | | |
| | | RR | P | I | N | P | I | N |
| AIDS | 242 | 241[a] | 236 | 6[a,b] | 0 | 241 | 1 | 0 |
| High Risk | 431 | 431 | 429 | 2[c] | 0 | 431 | 0 | 0 |
| Total | 673 | 672 | 665 | 8 | 0 | 672 | 1 | 0 |

a. One OraSure sample was EIA false negative, Western blot indeterminate (gp160+, p24±); the matching serum sample was EIA repeatedly reactive and Western blot positive (gp160+, pg120+, p65+, p51+, p24+).

b. One of the six Western blot indeterminate OraSure samples was repeatedly reactive on EIA and concordant on Western blot with an indeterminate result for the matching serum specimen. The remaining five OraSure specimens were discordant (indeterminate) with the matching serum specimens (positive) due to the required cardinal bands being visible but of insufficient intensity to be scored as Present.

c. One OraSure sample was discordant due to the intensity of bands on the OraSure Western blot (gp160+, gp120±, p65±, p51±, gp41±). The banding pattern of the second indeterminate OraSure sample was gp160+, gp120±. The matching serum specimens' banding patterns were gp160+, gp120+, p65+, p51±, gp41+, p24±, p18±, and gp160+, gp120+, p65±, gp41+, p24±, p18±, respectively.

RR = Repeatedly Reactive; P = Positive; I = Indeterminate; N = Negative.

In this study, the sensitivity of the OraSure Western blot testing of oral specimens from the 242 confirmed positive AIDS subjects was 97.5% (236/242) with 2.5% (6/242) indeterminate, and from the 431 confirmed high risk subjects was 99.5% (429/431) with 0.5% (2/431) indeterminate, with no OraSure Western blot false negatives in either group. All OraSure indeterminate blots showed the gp160 band as present and at least one additional cardinal band (gp120, gp41, p24) as visible, but of insufficient intensity to be called present. One of the OraSure indeterminate blots corresponded to the Western blot indeterminate serum specimen.

### Specificity Studies

The performance of the OraSure Western blot in documented seronegative subjects was evaluated by testing specimens from 495 EIA negative subjects (using oral fluid) at low risk for HIV-1 infection, 14 EIA repeatedly reactive specimens found by screening 1,887 persons at low risk for HIV-1 infection, 248 subjects with non-HIV related medical conditions (non-specificity subjects), and 267 specimens from high risk seronegative subjects. Thus, a total of 1,024 OraSure HIV-1 Western blots and serum Western blots were performed on these individuals. The results of this testing are presented in Table 3.

**Table 3. Comparative Study of Western Blot Results in Low Risk, Non-Specificity, and High Risk, HIV-1 Negative Populations**

| EIA Result for OraSure Specimens | OraSure HIV-1 Western Blot Interpretation | Licensed Serum HIV-1 Western Blot Interpretation | | | Totals |
|---|---|---|---|---|---|
| | | Positive | Ind. | Negative | |
| EIA Negative (n=1,007) | Positive | 0 | 0 | 0 | 0 |
| | Indeterminate | 0 | 96 | 111 | 207 |
| | Negative | 0 | 295 | 505 | 800 |
| EIA RR (n=17) | Positive | 0 | 0 | 0 | 0 |
| | Indeterminate | 0 | 3 | 1 | 4 |
| | Negative | 0 | 7 | 6 | 13 |
| Totals | | 0 | 401 | 623 | 1,024 |

Seventeen OraSure specimens (14 low risk and 3 high risk) were EIA repeatedly reactive. Thirteen of the 17 EIA false positive specimens were correctly identified as negative by the OraSure HIV-1 Western blot. Thus 2,893 out of 2,897 subjects (99.9%) were correctly identified as HIV-1 antibody negative by a combination of EIA and Western blot testing of OraSure samples. The four remaining specimens were indeterminate by OraSure HIV-1 Western blot (two of the four due to non-viral bands only). The indeterminate rate for uninfected persons who are EIA repeatedly reactive by OraSure was 23.5% (4/17) as compared to 58.8% (10/17) for serum.

Western blot was also performed on serum and OraSure specimens from 1,007 EIA negative subjects (using oral fluid). This testing identified 20.6% of OraSure specimens and 39.2% of sera as indeterminate. The overall concordance between the two types of specimens was 59.6%. Differences were largely due to non-viral bands that were present for one type of sample but not the other for individual subjects.

### Analytical Sensitivity

#### *Titration of Matching OraSure and Serum Specimens*

Fifteen randomly selected matching OraSure and serum repository specimens that had been obtained from HIV-positive individuals were titrated. Titrated serum specimens were tested with the licensed serum HIV-1 Western Blot Kit and matching titrated OraSure specimens were tested in parallel with the OraSure HIV-1 Western Blot Kit. The assay endpoint in this study was the last dilution at which a positive Western blot result was observed for each specimen. The results of this study are shown in Table 4.

Endpoints were obtained for all serum specimens and for 14 of the 15 OraSure specimens tested. The one specimen not yielding an endpoint (ID# 19052) had an indeterminate result when the neat OraSure specimen was tested. This assignment was based on positive reactivity for the gp160 and gp120 bands, and an indeterminate reactivity for the gp41 band. The corresponding serum specimen for subject 19052 also had a comparatively low titer (1:4). A review of medical records revealed that this individual was severely immunocompromised at the time of specimen acquisition ($CD4^+$ count = 18/$mm^3$).

The average ratio of the serum endpoints to the OraSure endpoints was 5.7:1. The difference in analytical sensitivity between the licensed serum Western Blot Kit and the OraSure Western Blot Kit ranged from a ratio of 12.8 to 0.40.

**Table 4. Highest Dilution Yielding Positive Western Blot Results for 15 Matching OraSure and Serum Specimens**

| Specimen Number | Highest OraSure Dilution[a] | Highest Serum Dilution[a] | Ratio[b] |
|---|---|---|---|
| 19026 | 1:20 | 1:256 | 12.8 |
| 19027 | 1:20 | 1:256 | 12.8 |
| 19078 | 1:20 | 1:256 | 12.8 |
| 19095 | 1:20 | 1:256 | 12.8 |
| 19105 | 1:5 | 1:64 | 12.8 |
| 19079 | 1:10 | 1:64 | 6.4 |
| 19032 | 1:10 | 1:16 | 1.6 |
| 19059 | 1:10 | 1:16 | 1.6 |
| 19066 | 1:50 | 1:64 | 1.28 |
| 19069 | 1:50 | 1:64 | 1.28 |
| 19080 | 1:250 | 1:256 | 1.02 |
| 19044 | 1:5 | 1:4 | 0.8 |
| 19101 | 1:100 | 1:64 | 0.64 |
| 19033 | 1:10 | 1:4 | 0.40 |
| **Mean ratio** | | | 5.7 |
| 19052 | Neat[c] | 1:4 | - |

a. Beyond standard specimen dilution per assay protocol.
b. Ratio of serum endpoint dilution/OraSure endpoint dilution.
c. Specimen #19052 was indeterminate (with viral bands) when the undiluted OraSure specimen was tested.

### *Titration of OraSure Seroconversion Specimens*

Repository OraSure and plasma specimens from an earlier seroconversion study were used to assess the analytical sensitivity of the OraSure HIV-1 Western Blot.

OraSure specimens were diluted and each dilution was assayed by EIA and Western blot. The objective of this study was to determine the highest dilution of the OraSure specimen that would produce a repeatedly reactive EIA result, based on product insert criteria, and would demonstrate viral band reactivity in the OraSure HIV-1 Western Blot Kit.

Table 5 shows the results of this testing. For each of the four time points, the OraSure Western Blot demonstrated viral bands at dilutions which produced non-reactive EIA results, yielding an average of ≥10-fold enhanced sensitivity over the EIA for OraSure specimens.

**Table 5. Reactivity of OraSure HIV-1 Seroconversion Specimens in the Oral Fluid Vironostika HIV-1 Microelisa System and the OraSure HIV-1 Western Blot Kit**

| ID# | Highest EIA Dilution | Highest WB Dilution | Blot to EIA Sensitivity |
|---|---|---|---|
| 01SC052591 | Negative at Neat | Positive at 1:10* | ≥ 10 |
| 01SC052691 | RR at Neat | Positive at 1:5* | ≥ 5 |
| 01SC052791 | RR at Neat | Positive at 1:5* | ≥ 5 |
| 01SC052991 | RR at Neat | Positive at 1:20* | ≥ 20 |

* Indicates the highest dilution tested  RR = Repeatedly Reactive

## Reproducibility

The reproducibility of the OraSure HIV-1 Western Blot Kit was evaluated at three separate test laboratories. The study included testing a three-member panel of pooled OraSure specimens with the OraSure HIV-1 Western Blot Kit. The OraSure reproducibility panel consisted of an HIV-1 antibody positive specimen, an HIV-1 antibody negative specimen, and an HIV-1 Western blot indeterminate specimen.

The panel members were tested on three separate days, using three separate OraSure HIV-1 Western Blot Kit production lots, resulting in a total of 27 test results being generated for each panel member. The percentage of times each band was scored reactive is presented in Table 6.

**Table 6. Reproducibility of the OraSure HIV-1 Western Blot Kit**

| Specimen # | Specimen Reactivity | Percent Frequency of Visible Bands[c] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | gp160 | gp120 | p65 | p55 | p51 | gp41 | p31 | p24 | p18 |
| 1 | Positive[a] | 100 | 100 | 100 | 0 | 100 | 100 | 100 | 100 | 18.5 |
| 2 | Negative | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | Indeterminate[b] | 100 | 100 | 100 | 0 | 100 | 100 | 3.7 | 70.4 | 0 |

a. Positive specimen known banding pattern: reactivity for gp160, gp120, p65, p51, gp41, p31, p24 and no reactivity for p55, p18.
b. Indeterminate specimen known banding pattern: reactivity for gp160, gp120, p65, p51, gp41, p24; and no reactivity for p55, p31, p18.
c. Frequency of visible bands (either Indeterminate or Present).

The results demonstrate that for positive specimens, negative specimens, and indeterminate specimens with known banding patterns, reproducibility is high.

### Reactivity in Other Disease Conditions

Matching OraSure and serum specimens were obtained at three sites from 248 subjects who were enrolled in the clinical trial because they had non-HIV-1 medical conditions that might result in antibodies cross-reactive with HIV-1 proteins or other potentially interfering factors. Specimens studied included 89 from multiparous women, 69 from subjects with non-HIV viral infections, 50 receiving anticoagulation therapy, 26 with autoimmune diseases other than AIDS, 11 with oral pathology, and 3 with polyclonal or monoclonal gammopathy. Although bands were present at viral band locations for four samples (1.6%), none of the strips could be interpreted as positive. Results are presented in Table 7.

**Table 7. Results of OraSure Western Blot Testing on Samples from Subjects with Non-HIV Disease Processes or Other Potentially Interfering Factors**

| OraSure HIV-1 WB Result | Band Specificities (# and % of samples) | | | | | | | | | Non-viral |
|---|---|---|---|---|---|---|---|---|---|---|
| | gp160 | gp120 | p65 | p55 | p51 | gp41 | p31 | p24 | p18 | |
| NEG 186 (75.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| IND 62[a] (25.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (3.2) | 0 (0.0) | 2 (3.2) | 1 (1.6) | 58 (93.5) |
| Total 248 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (0.8) | 0 (0.0) | 2 (0.8) | 1 (0.4) | 58 (23.4) |

a. 58 of 62 results Indeterminate due to non-viral bands only.

Testing of specimens from this population revealed that the number of OraSure indeterminates (62; 25.0%) was substantially less than the number of serum indeterminates (121; 48.8%). The number of serum indeterminates due to the presence of viral bands (27; 10.9%) was substantially greater than the number of OraSure indeterminates due to the presence of viral bands (4; 1.6%).

**Summary**

In this clinical trial using the recommended OraSure algorithm, 3,558/3,570 subjects received the correct HIV-1 antibody results from a single OraSure sample the first time it was tested. In 11 of the remaining subjects, the Western blot was indeterminate: for these 11, the algorithm would lead to appropriate follow-up testing. Thus, in 3,569/3,570 (99.97%) subjects either the correct result was reached or appropriate follow-up testing would be triggered. It is concluded that OraSure testing is a highly accurate alternative to serum testing.

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## KIT AVAILABILITY

**OraSure® HIV-1 Western Blot Kit**

20 Tests

Product Number 501-0000

**Manufactured by**

OraSure Technologies, Inc.

220 East First Street
Bethlehem, PA 18015
www.orasure.com

For technical assistance,
contact OraSure Technologies' Technical Service Department
at 1-800-672-7873

Printed in USA

Item# 3001-0792 (Rev. 11/07)

# ATTACHMENT 5

BIO-RAD

# HUMAN IMMUNODEFICIENCY VIRUS TYPE I

## GS HIV-1 Western Blot

A Western Blot Kit for the Detection of Antibodies to Human Immunodeficiency Virus Type 1 (HIV-1) in Human Serum, Plasma or Dried Blood Spots.

For *in vitro* diagnostic use

32508 • 40 Tests

For Reference Use Only

**FOR REFERENCE USE ONLY: DO NOT USE In place of package Inserts provided with each test kit.**

For Reference Use Only

## NAME AND INTENDED USE

The GS HIV-1 Western Blot Kit is an in vitro qualitative assay for the detection and identification of antibodies to Human Immunodeficiency Virus Type 1 (HIV-1) in human serum, plasma, or dried blood spots. It is intended for use with persons of unknown risk as an additional, more specific test on human serum, plasma, or dried blood spot specimens found to be repeatedly reactive using a screening procedure, such as Enzyme-Linked Immunosorbent Assay (ELISA), and as an additional, more specific test for use with serum, plasma, or dried blood spot specimens obtained from subjects found to be reactive using rapid HIV-1 tests.

## SUMMARY AND EXPLANATION OF THE TEST

The major etiologic agent of Acquired Immunodeficiency Syndrome (AIDS) is a retrovirus called Human Immunodeficiency Virus Type 1 (HIV-1).[1-3] Enzyme immunoassays (EIA) to detect the presence of viral-specific antibodies to HIV-1 have been described by several investigators. Patients with AIDS and AIDS-related conditions exhibit a high prevalence of antibodies to HIV-1, and antibodies to HIV-1 have also been reported in virus positive, asymptomatic individuals.[4,5]

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The original purpose of HIV-1 screening assays was to detect potentially infectious units of blood and to prevent these units from being used in transfusion or in the manufacture of blood products for transfusion. However, these highly sensitive tests have a relatively low positive predictive value for populations with a low prevalence of HIV-1 infection.[6] Some specimens may contain antibodies to HLA Class II histocompatibility antigens found on certain cell lines used to produce the virus for commercial applications, or specimens may react with bacterial contaminants associated with production of recombinant proteins.[7,8] Other individuals who have had no known exposure to HIV-1 react with HIV-1 core proteins in the EIA for unknown reasons.[9] Since the psychosocial and medical implications of a positive antibody test may be devastating, it is prudent to perform additional testing on such samples to further demonstrate the presence of antibodies specific to HIV-1.

The Western blot as described by Tsang, et. al.,[10] is useful for elucidating the specificity of the antibody response to HIV-1.[11-13] In the Western blot assay, disrupted proteins of HIV-1 are fractionated by electrophoresis according to molecular weight using a polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS). The resolved protein bands are transblotted to a nitrocellulose sheet. The nitrocellulose sheet is then cut into strips, which are reacted with serum, plasma, or dried blood spot specimens.

If virus-specific antibodies are present, they bind to their corresponding viral protein bands. The bands are visualized by using a phosphatase-labeled goat anti-human immunoglobulin conjugate, followed by a substrate for the enzyme. The presence of specific HIV-1 immunoglobulins in serum specimens is indicated by labeling of HIV-1-specific proteins on the strip. Recognized HIV-1 viral antigens produce bands at gp160, gp120, p65, p55, p51, gp41, p40, p31, p24, and p18.

A sample that is repeatedly reactive in the EIA and POSITIVE on the Western blot is presumed to be positive for antibodies to HIV-1.[14] Individuals with positive tests should be referred for medical evaluation. A diagnosis of AIDS can be made only if an individual meets the case definition of AIDS established by the Centers for Disease Control.

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## BIOLOGICAL PRINCIPLES OF THE PROCEDURE

Purified, inactivated HIV-1 strain LAV grown in the CEM cell line is disrupted and electrophoretically resolved into bands. The proteins are transblotted onto nitrocellulose sheets, which are cut into strips. Samples are diluted in Specimen Diluent/Wash and applied to the nitrocellulose strip. If specific HIV-1 antibody is present, it binds to proteins resolved on the strip. Unbound sample is removed by washing. The phosphatase-labeled conjugate is then added to the strip and allowed to incubate. The conjugate attaches to antibody already bound to viral proteins on the strip. Excess conjugate is removed by washing. Color Development Reagent is then added to the strip. Reaction sites, where enzyme-labeled antibody is bound, are identified by purple bands. The position and intensity of the bands are compared to reference strips developed using Positive Control sera.

# REAGENTS

## GS HIV-1 Western Blot Product Description
## Product No: 32508 (40 Tests)

| Component | Contents | Preparation |
|---|---|---|
| R1 • HIV-1 Western Blot Strips (40)<br>2 packages of 20 strips | • Package contains 20 strips, sufficient for 20 tests; Nitrocellulose strips preblotted with resolved HIV-1 viral proteins<br>• Blotting paper buffer contains 0.1% sodium azide and 0.1% ProClin 300 | Ready to use as supplied. |
| C0 • Western Blot Negative Control<br>1 vial (0.2ml) | • Normal human serum or plasma<br>• Non-reactive for HBsAg and antibodies to HIV, HCV and HTLV-I/II<br>• 0.1% sodium azide<br>• 0.5% ProClin 300 | Dilute in Working Specimen Diluent/Wash as described. |
| C1 • HIV-1 Western Blot Low Positive Control<br>1 vial (0.2ml) | • Heat inactivated human serum or plasma containing antibodies reactive to HIV-1<br>• Non-reactive for HBsAg<br>• Non-reactive for antibody to HCV and HTLV-I/II<br>• 0.1% sodium azide<br>• 0.5% ProClin 300 | Dilute in Working Specimen Diluent/Wash as described. |
| C2 • HIV-1 Western Blot High Positive Control<br>1 vial (0.2ml) | • Heat inactivated human serum or plasma containing antibodies reactive to HIV-1<br>• Non-reactive for HBsAg<br>• Non-reactive for antibody to HCV and HTLV-I/II<br>• 0.1% sodium azide<br>• 0.5% ProClin 300 | Dilute in Working Specimen Diluent/Wash as described. |
| R2 • Western Blot Specimen Diluent/Wash (5X)<br>2 bottles (100ml) | • TRIS buffered saline<br>• Milk Proteins<br>• 0.5% ProClin 300 | Dilute in deionized water as described |
| R3 • HIV-1 Western Blot Conjugate<br>1 bottle (80ml) | • Anti-human IgG, IgA and IgM (Goat) alkaline phosphatase conjugated solution<br>• 0.1% sodium azide<br>• 0.5% ProClin 300 | Ready to use as supplied. |
| R4 • Western Blot Color Development Reagent<br>1 bottle (100ml) | • 5-bromo-4-chloro-3-indolyl phosphate (BCIP)<br>• Nitro blue tetrazolium (NBT)<br>• Organic base/TRIS Buffer | Ready to use as supplied. |
| Disposable Reaction Trays<br>8 trays | • Disposable, slotted reaction trays<br>• Lids | Ready to use as supplied. |

For Reference Use Only

Store the kit at 2-8°C upon arrival. All components should be stored at 2-8°C and returned to 2-8°C after use. Bring 5X Specimen Diluent/Wash to room temperature(15-30°C) before use. Return unused strips to package and reseal.

## WARNINGS FOR USERS
### For *in vitro* Diagnostic Use

**WARNING: FDA has licensed this test for use with serum, plasma, and dried blood spot specimens only. Use of this licensed test kit with specimens other than those specifically approved for use with this test kit may result in inaccurate test results.**

1. This test kit should be handled only by qualified personnel trained in laboratory procedures and familiar with their potential hazards. Wear appropriate protective clothing, including lab coat, eye/face protection and disposable gloves (synthetic, non-latex gloves are recommended) and handle with the requisite Good Laboratory Practices. Wash hands thoroughly after performing the test.
2. Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.
3. Do not pipette by mouth.
4. No known test method can offer complete assurance that infectious agents are absent. Therefore, all human blood derivatives, reagents and human specimens should be handled as if capable of transmitting infectious disease. It is recommended that reagents and human specimens be handled in accordance with the OSHA Standard on Bloodborne Pathogens.[15] Biosafety Level 2[16] or other appropriate biosafety practices[17,18] should be used for materials that contain or are suspected of containing infectious agents. The following human blood derivatives are found in this kit:
    a. The Nitrocellulose strips are preblotted with resolved HIV-1 viral proteins.
    b. The Normal Human source material (serum or plasma) used in the preparation of the Negative Control (C0) has been tested and found nonreactive for hepatitis B surface antigen (HBsAg) and antibodies to hepatitis C virus (HCV) and human immunodeficiency viruses (HIV-1 and HIV-2).

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c. Human source material containing antibodies reactive to HIV-1, used in the preparation of the Positive Controls (C1 and C2) has been heat-treated. It has been tested and found nonreactive for hepatitis B surface antigen (HBsAg) and antibody to hepatitis C virus (HCV).

5. Biological spills: Human source material spills should be treated as potentially infectious.

   Spills not containing acid should be immediately decontaminated, including the spill area, materials and any contaminated surfaces or equipment, with an appropriate chemical disinfectant that is effective for the potential biohazards relative to the samples involved (commonly a 1:10 dilution of household bleach, 70-80% Ethanol or Isopropanol, an iodophor [such as 0.5% Wescodyne Plus, EPA Registration # 4959-16-52], or a phenolic, etc.), and wiped dry.[19-21]

   NOTE: DO NOT PLACE SOLUTIONS CONTAINING BLEACH INTO THE AUTOCLAVE.

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6. The following is a list of potential chemical hazards contained in some kit components (see Section 4 - REAGENTS):

   a. ProClin® 300 (0.1% or 0.5%), a biocidal preservative that is irritating to eyes and skin, may be detrimental if enough is ingested, and may cause sensitization by skin contact; prolonged or repeated exposure may cause allergic reaction in certain sensitive individuals.

   b. 0.1% Sodium azide ($NaN_3$), a toxic biocidal preservative, which may be harmful in contact with skin or if swallowed; it has been evident to kill at low concentrations, if enough is ingested. Sodium azide may react with certain metals, including lead or copper often found in plumbing, to form highly explosive metal azides. If solutions containing dilute azide are disposed of in the sink after inactivation, flush with copious water to prevent potential explosive build-up.

   The Material Safety Data Sheets are available on request.

7. Dispose of all specimens and material used to perform the test as though they contain an infectious agent. Laboratory, chemical or bio-hazardous wastes must be handled and discarded in accordance with all local, regional and national regulations.

## PRECAUTIONS FOR USERS

1. Do not use the kit beyond the stated expiration date.
2. Bring Specimen Diluent/Wash to room temperature before use.
3. The only reagents that may be used with different lots of the GS HIV-1 Western Blot are the Specimen Diluent/Wash and Color Development Reagent. Do not mix any other reagents from different lots.
4. Exercise care in opening and removing aliquots from vials to avoid microbial contamination.
5. Prior to removing a strip from the pouch, clean the work surface and forceps with 70% ethanol or isopropyl alcohol.
6. Using forceps, remove each strip from the left side of the series in the package first, and maintain the same order in the reaction trays.
7. For the pipetting of controls and specimens, use individual pipette tips to eliminate carryover of samples.
8. Before measuring samples or reagent, pre-wet the pipette tips with the sample or reagent.
9. Avoid cross-contamination during sample incubation and aspiration:
    - Be particularly careful when transferring reaction trays to the rotator (during sample incubation) to avoid spilling fluid from the troughs.
    - Liquid in the troughs should not contact the tray lid during rotation.
    - A strip may be placed in every second trough to further reduce the risk of cross-contamination.
    - Use clean tips to aspirate samples to avoid cross-contamination.

For Reference Use Only

10. Do not re-use trays or lids.
11. It is important that the pipette used to dispense Working Specimen Diluent/Wash be scrupulously clean. A disposable pipette is preferable.
12. Use a clean, disposable container (e.g. reagent reservoir) and pipette for dispensing Conjugate. Exposure of Conjugate to serum will inactivate Conjugate.
13. It is imperative that the final wash, prior to the addition of Color Development Reagent, be performed with deionized or distilled water. Residual Specimen Diluent/Wash in the troughs may decrease band intensity by inhibiting the substrate reaction.
14. Handle negative and positive controls in the same manner as patient specimens.
15. If a specimen is inadvertently not added to a strip, the assay result will read negative.
16. Inadequate adherence to package insert instructions may result in erroneous results.

For Reference Use Only

## REAGENT PREPARATION AND STORAGE

### A. HIV-1 Western Blot Strips

HIV-1 Western Blot Strips are packed in a resealable plastic pouch between buffer-soaked blotting paper. When ready to use, cut the bag *below* the seal line and retain the upper portion of the bag. Remove the strips from the bottom portion of the bag. Separate the strips to be assayed and place them into troughs of a reaction tray. Place any remaining strips (still inside blotting paper) in the upper portion of the bag. Seal the bag using the zip closure. Keep tightly sealed. DO NOT LET STRIPS DRY OUT. Store at 2-8°C.

**Note:** Avoid contaminating the strips or moist blotting paper during handling. This may cause false reactivity.

## B. Working Specimen Diluent/Wash

Use Working Specimen Diluent/Wash for wash solution and dilution of serum and plasma samples or elution of dried blood spots. Prepare Working Specimen Diluent/Wash as follows: bring 5X Specimen Diluent/Wash to room temperature, invert to mix before using, and then dilute 1:5 with deionized or distilled water (i.e. 1 part 5X Specimen Diluent/Wash plus 4 parts deionized or distilled water). Clinical laboratory reagent water Type I or Type II is acceptable. For each strip to be tested, prepare 15 ml (i.e., 3 ml 5X Specimen Diluent/Wash plus 12 ml deionized water). Prepare the volume of diluted reagent that will be adequate for the entire run. Mix working solution when combined and again just prior to use. The Working Specimen Diluent/Wash may be stored at 2-8°C for up to two weeks. Bring to room temperature and mix thoroughly prior to use.

### Preparation of Working Specimen Diluent/Wash

| Number of Strips to be used | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Amount of 5X Spec. Dil/Wash (ml) | 12 | 15 | 18 | 21 | 24 | 27 | 30 | 33 | 36 | 39 | 42 | 45 | 48 | 51 | 54 | 57 | 60 |
| Deionized Water (ml) | 48 | 60 | 72 | 84 | 96 | 108 | 120 | 132 | 144 | 156 | 168 | 180 | 192 | 204 | 216 | 228 | 240 |
| Total Volume (ml) | 60 | 75 | 90 | 105 | 120 | 135 | 150 | 165 | 180 | 195 | 210 | 225 | 240 | 255 | 270 | 285 | 300 |

For Reference Use Only

## SPECIMEN COLLECTION, PREPARATION AND STORAGE

### Serum or Plasma

Serum or plasma may be used. The following anticoagulants have all been evaluated and found to be acceptable: EDTA, heparin, sodium citrate, CPDA-1, and ACD. Samples which are collected into anticoagulant tubes should be filled as labeling indicates to avoid improper dilution. Specimens with observable particulate matter should be clarified by centrifugation prior to testing. No clinically significant effect has been detected in assay results with increased levels of protein, lipids, bilirubin, or hemolysis, or after heat inactivation of patient samples.

Serum or plasma may be stored at 2-8°C for up to seven days. Samples should not be used if they have incurred more than 5 freeze/thaw cycles. Mix samples thoroughly after thawing.

*If specimens are to be shipped, they should be packed in compliance with Federal Regulations covering the transportation of etiologic agents.* Studies have demonstrated that specimens may be shipped refrigerated (2-8°C) or at ambient temperatures (≤ 37°C) for up to 7 days. For shipments that are in transit for more than 7 days, specimens should be kept frozen (-20°C or lower). Refrigerate samples at 2-8°C at receipt, or freeze for longer storage.

**Collection of Dried Blood Spots**

In addition to detecting antibodies to Human Immunodeficiency Virus Type 1 (HIV-1) in human serum or plasma, the GS HIV-1 Western Blot may be used to test whole blood specimens collected onto filter paper and dried. Drops of whole blood should be obtained by using a licensed collection kit or collected according to the National Committee for Clinical Laboratory Standards,[18] by either finger puncture or heel stick.

For Reference Use Only

1. Label a separate piece of filter paper for each specimen with the appropriate specimen identification. Use a ball point pen or other water-indelible marker. Handle the filter paper by the edges; do not touch the areas that will be used to collect specimens.

2. Prepare the area (either finger or heel) for puncture. The puncture must be performed with sufficient force and penetration to sustain a flow of at least several drops of blood. Allow a large drop of free flowing blood to collect at the puncture site. Touch the filter paper to the edge of the drop to collect the drop, and allow another large drop to form at the puncture site. Continue to collect drops in this manner until the wound ceases to bleed or until collection is sufficient.

3. Collect each drop of blood in a separate area of the filter paper (if the paper is marked with several circles, place each drop in a different circle). Do not layer successive drops of blood in the same spot. In addition to the sample that is required to perform the EIA (i.e., one spot of blood ≥ 1/4 of an inch in diameter), at least one spot of blood that is

≥ 1/4 of an inch in diameter must be obtained in order to perform the GS HIV-1 Western Blot.

4. If the wound stops flowing before sufficient blood has been obtained, a second puncture should be performed. The wound may be massaged very gently to encourage formation of large blood droplets. Do not squeeze the wound to obtain more blood as this may result in hemolysis of the specimen or a mixture of other body fluids with the specimen.

5. After the blood has been absorbed into the filter paper, it should be dried at room temperature for at least three hours. The filter paper may be allowed to dry at room temperature overnight. When dry, the spots will be a uniform dark brown. No areas of red coloration should be seen; the appearance of the spots should be similar to that of a dried blood stain.

6. When the blood spots are completely dry, a sample may be punched and eluted as described below (See Specimen Handling and Preparation). If dried blood specimens are to be shipped, they should be enclosed and sealed in either a moisture barrier container, such as a heavy duty zip-lock bag with desiccant,[22] or a high-quality bond envelope.[23]

For Reference Use Only

**Storage of Dried Blood Spots**

Studies performed indicate that completely dried specimens may be stored frozen (-20°C) or refrigerated (2-8°C) for at least two months under low humidity conditions. Storage under accelerated temperature conditions (room temperature or 37°C) leads to increasing non-specific reactivity over time. Therefore, it is recommended that dried blood spots be stored for no more than one week at room temperature, or up to two months refrigerated or frozen; routine storage at room temperature or higher is not recommended. If specimens are stored at any conditions other than the ones listed above, the user must validate the stability of the specimens under those storage conditions. If specimens are to be stored in a humid environment (≥ 60% relative humidity), the user should include a desiccant.

This kit is not licensed for use with specimens other than serum, plasma, and dried blood spots. This kit is not intended for use on saliva/oral fluids or urine samples.

## GS HIV-1 WESTERN BLOT PROCEDURE

### Materials Provided

See Reagents Section on Page 3.

### Materials Required But Not Provided

1. Disposable Pipettes.
2. Calibrated precision micropipettes to deliver variable volumes from 5 - 1000 µl, (accurate within ± 5%).
3. Disposable pipette tips.
4. Rotary platform capable of rotating at 50 - 60 rpm.
5. Aspirator.
6. Plastic forceps for strip handling.
7. Glass or plastic container for preparation of Working Specimen Diluent/Wash, 15 - 600 ml.
8. Clean, disposable container, such as a reagent reservoir, for dispensing Conjugate.
9. Deionized or distilled water. Clinical laboratory reagent water Type I or Type II is acceptable.
10. Gloves.
11. Laboratory timer.

For Reference Use Only

**Preliminary Statements**

1. The expected run time for this procedure is approximately 3 hours. Each run of this assay must proceed to completion without interruption after it has been started.

2. Positive and Negative Controls must be evaluated with each run. Compare intensity of patient samples to controls for each set of strips to determine patient results.

3. **The minimum controls to be included in each run of this assay are: one HIV-1 Low Positive Control, one HIV-1 High Positive Control, and one Negative Control. If multiple packages of strips are used in one run, controls must be included for each package of strips.**

4. Do not splash controls, specimens, or reagents between troughs of the reaction tray.

5. Cover reaction trays for each incubation step using lid provided, or equivalent.

6. Ensure that each one of the strips is entirely covered with liquid during the incubation steps.

7. Adhere to the recommended time constraints for the incubation steps.

For Reference Use Only

## SPECIMEN HANDLING AND PREPARATION

**Caution: Handle all specimens, controls, and HIV-1 Western Blot Strips as though capable of transmitting infectious disease.**

**Dried Blood Spot Specimen Preparation**

1. Use a 1/4" paper punch to remove a 1/4" disk of each whole blood specimen to be tested. Punch the disk from a uniform area of one of the completely dried spots of blood. Place each 1/4" disk in a separate, clean, empty trough of a reaction tray.

2. Use a precision pipette or repeating dispenser to add 500 µl of Working Specimen Diluent/Wash to each trough containing a filter paper disk. Mix the specimens well to wet the filter paper thoroughly (for

example, use a rotator). Cover the reaction tray with a lid to minimize evaporation. Tilt tray approximately 5/8" (15 mm) to concentrate liquid in area of spot. Elute the specimens 2 hours at room temperature with rotation at 50 - 60 rpm, or overnight (static) at 2-8°C.

3. At the end of the two hour elution or when the refrigerated eluates warm to room temperature, the filter paper disk should be almost white (a faint brown color may remain in the disk). Specimens eluted in this manner are ready to use without further dilution in the GS HIV-1 Western Blot. The filter paper disk may be removed from the trough with clean forceps before starting the assay, or the filter paper disk may be left in the trough and removed just prior to the addition of Conjugate (before step 7 of the Procedure). Clean the forceps with deionized water and wipe dry in between removal of each filter paper disk in order to avoid cross-contamination.

**Wash Procedure for Serum, Plasma or Dried Blood Spots**

Repeat this procedure four times after the primary (sample) and secondary (conjugate) incubations. Incomplete or ineffective washing will compromise the assay. **DO NOT ALLOW THE STRIPS TO DRY OUT.**

For Reference Use Only

1. **Aspirate the liquid from the troughs.** Wash aspiration manifold (if used) by aspirating water for 3 - 5 seconds between trays of strips. **Immediately fill each trough** with at least 1 ml Working Specimen Diluent/Wash.

2. **Rock the reaction tray(s)** back and forth approximately 8 times. Be certain that each strip is immersed in liquid and is moving freely when rocked.

3. **Aspirate the liquid completely.** Wash aspiration manifold (if used) by aspirating water for 3 - 5 seconds between trays of strips.

## ASSAY PROCEDURE

1. Prior to the start of the assay, prepare Working Specimen Diluent/Wash (see page 8, step B). **Allow to come to room temperature before use.**

2. Label the reaction trays appropriately to maintain control and sample identification.

3. **Add diluted specimens to reaction trays.**

   - **Serum and Plasma:** Using a clean, disposable pipette, carefully add 1 ml Working Specimen Diluent/Wash into each trough. Using a clean, disposable, precision micropipette, transfer 10 µl of each control or specimen to the respective trough containing 1 ml of Working Specimen Diluent/Wash (final dilution 1:101).

   - **Dried Blood Spots:** See Dried Blood Spot Specimen Preparation, page 12. Note: the required volume of dried blood spot eluate is 0.5 ml/strip.

4. **Place one strip, with the *indicator line facing up,* into each trough containing a diluted serum or plasma specimen, dried blood spot eluate, or control.**

   Note: To avoid cross-contamination of the strips, the following instructions should be followed.

   a. While wearing a fresh pair of gloves, cut the strip pouch below the heat seal with cleaned scissors, leaving the zipper seal intact.
   b. Trim the side edges of the strip pouch around the filter paper, leaving the bottom (folded) edge intact.
   c. Do not touch the filter paper with anything but cleaned forceps. Leave the outer plastic in place to hold the pouch, and use cleaned forceps when manipulating filter paper and strips.
   d. Gently detach each strip with forceps at the top of the strip (near the number).

For Reference Use Only

e. Transfer the strip carefully; avoid contact with contaminated surfaces. Do not touch the forceps to the liquid in the trough.

f. Keep unused strips and filter paper in the original plastic folder. Store the plastic folder in the zipper sealed bag.

5. Cover the reaction tray with the lid and **incubate with gentle agitation** on a rotator at 50-60 rpm for 60-65 minutes at room temperature.

   Note: Be certain that each strip is immersed in the liquid and is moving freely during rotation. Liquid should not contact the tray lid during rotation.

6. After incubation, aspirate the liquid from each trough. **Wash the strips at least four times** with Working Specimen Diluent/Wash (refer to the Wash Procedure). After the final wash, add 1.0 ml Working Specimen Diluent/Wash to each trough. Place the reaction tray on the rotator for 4-6 minutes.

   Note: If filter paper disks have not already been removed, remove them now with forceps. Clean the forceps with deionized water and wipe dry between removal of each filter paper disk in order to avoid cross contamination.

For Reference Use Only

7. Following the soak, aspirate the contents of each trough completely. ***Immediately*** **add 1.0 ml Conjugate to each trough.**

8. Cover the reaction tray with the lid and **incubate with gentle agitation** on the rotator at 50-60 rpm for 45-50 minutes at room temperature.

   Note: Be certain that each strip is immersed in the liquid and is moving freely during rotation. Liquid should not contact the tray lid during rotation.

9. After incubation, aspirate the liquid from each trough. **Wash the strips at least four times** with Working Specimen Diluent/Wash (refer to the Wash Procedure). After the final wash, **add 1.0 ml deionized water** to each trough. Place the reaction tray on a rotator for 4-6 minutes.

10. Following the soak, aspirate the deionized water from each trough completely. ***Immediately*** **add 1.0 ml Color Development Reagent** to each trough. Gently rock the reaction tray back and forth at least twice. Be sure that each strip is immersed in Color Development Reagent. **Allow color to develop for 3-4 minutes. Do not over-incubate Color Development Reagent.**

11. **Stop the reaction** by aspirating the Color Development Reagent and flooding each trough with a minimum of 1.0 ml deionized water. Allow to stand for 5-10 minutes. Aspirate the water.

12. Allow the strips to **air dry** in the reaction tray before reading the bands. Handle the strips carefully. Use forceps to remove the strips from the troughs.

13. Store completed strips away from direct light to avoid fading. For permanent storage, it may be desirable to mount the strips on durable paper and cover them with a plastic sheet for protection.

For Reference Use Only

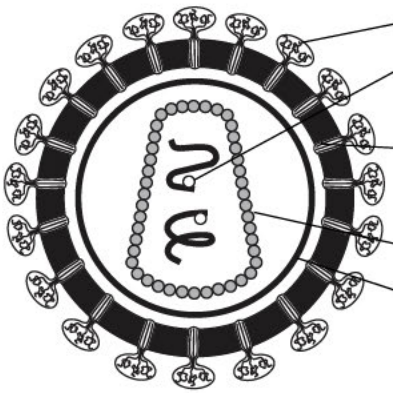
## QUALITY CONTROL

**For the results of the assay to be considered valid, the following conditions must be met:**

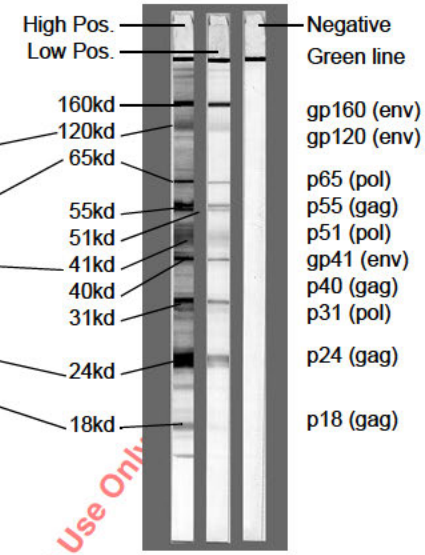
| | |
|---|---|
| **Negative Control** | No bands should be observed. |
| **HIV-1 Low Positive Control** | A band must be present at gp120, and the interpretation of the Low Positive Control must be positive. Other bands may or may not be present. |
| **HIV-1 High Positive Control** | Bands must be present at gp160, gp120, gp41 and p24. Other bands may or may not be present. |

The CDC/ASTPHLD criteria are used for result interpretation.[24] The major bands of diagnostic significance on a Western blot reactive for HIV-1 are gp160, gp120, gp41, and p24. Other bands may also be present. Note: the p51 and p55 bands may co-migrate and can be interpreted as one p51/p55 entity.

**ANATOMY OF HIV-1**



**HIV-1 ASSOCIATED BANDS**

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## INTERPRETATION OF RESULTS

Evaluate each strip for the presence of bands. A band is defined as a distinct purple line which extends horizontally across the strip where human antibodies have bound to resolved proteins. The lines may be of varying thicknesses. Smudges, spots, and lines which do not extend the width of the strip should not be interpreted as bands. Any lines that are higher than gp160 or lower than p18 on the strip are outside of the reading area of the strip and therefore are not to be interpreted as bands.

Use the HIV-1 High and Low Positive Controls to identify bands which may be present on patient specimen strips. Score relative intensity of

bands present on patient specimen strips by comparing to bands on control strips as follows:

| Intensity of Band | Score |
|---|---|
| • Absent | - |
| • Less than the intensity of gp120 on the HIV-1 Low Positive Control strip. | +/- |
| • At least as intensely reactive as gp120 on the HIV-1 Low Positive Control strip but less intense than gp120 on the HIV-1 High Positive Control strip. | + |
| • Greater than or equal to the intensity of gp120 on the High Positive Control strip. | ++ |

Interpret the immunoblot as NEGATIVE, INDETERMINATE, or POSITIVE based on the pattern that is present.

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| | |
|---|---|
| **POSITIVE** | At least TWO of the major bands: gp160 and/or gp120, gp41, or p24 must be present. (The presence of gp160 and/or gp120 qualifies as one major band.) Bands must be at least as intense as the Low Positive Control gp120 band (a reactivity score of + or greater) to be considered POSITIVE. The band at gp41 must be broad and diffuse. |
| **INDETERMINATE** | One or more bands are present but the blot does not meet the criteria for a POSITIVE result as described above. |
| **NEGATIVE** | No bands are present.* |

*Note: Negative dried blood spot specimens frequently exhibit a weakly reactive (+/-) fine band migrating within the wide gp41 region. This reactivity is clearly distinguishable from gp41, which is a broad, diffuse band. Dried blood spot specimens that are reactive only with this discrete "p42" band or contain only lines or marks that are higher than gp160 or lower than p18 may be interpreted as NEGATIVE.

INDETERMINATE results should not be considered either POSITIVE or NEGATIVE. Additional immunoblot testing and clinical evaluation must be utilized to correctly evaluate an INDETERMINATE result.

## LIMITATIONS OF THE PROCEDURE

**NOTE: Only a serum or plasma sample should be used to test blood or plasma intended for transfusion or further manufacture.**

1. The assay must be performed in strict accordance with these instructions to obtain reproducible results.
2. Although a persistently POSITIVE immunoblot for antibodies to HIV-1 indicates infection with the virus, a diagnosis of Acquired Immunodeficiency Syndrome or AIDS can only be made on clinical grounds if an individual meets the case definition of AIDS established by the Centers for Disease Control.[25]
3. Individuals with POSITIVE immunoblots for antibodies to HIV-1 should be referred for medical evaluation which may include additional testing. The clinical implications of antibodies to HIV-1 in an asymptomatic individual are not known. However, a large proportion of such individuals have virus detectable in their blood cells and some such individuals will develop immunodeficiency with the passage of time.[26]
4. It is generally recognized that detection of HIV antibody in infants born to seropositive mothers is not adequate to diagnose HIV infection in the infant, since maternal IgG frequently persists for as long as 18 months after birth. Supplemental assays designed specifically for neonatal specimens may be helpful in resolving such cases.[27]
5. INDETERMINATE immunoblots should not be used as the sole basis of diagnosis of HIV-1 infection. However, such findings may provide useful information in the context of medical evaluation in which clinical information is available.
6. Persons with an INDETERMINATE immunoblot should be retested using a fresh specimen after six months.

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7. A NEGATIVE immunoblot does not exclude the possibility of infection with HIV-1. Antibody testing should not be used in lieu of blood donor exclusion and self-exclusion procedures.

8. Strips which are obscured by the development of dark blotches or marks should not be interpreted.

9. A person who has antibodies to HIV-1 is presumed to be infected with the virus, except that a person who has participated in an HIV vaccine study may develop antibodies to the vaccine and may or may not be infected with HIV. Clinical correlation is indicated with appropriate counseling, medical evaluation and possibly additional testing to decide whether a diagnosis of HIV infection is accurate.

## PERFORMANCE CHARACTERISTICS

The performance of the GS HIV-1 Western Blot was evaluated in clinical studies in low risk, high risk, and AIDS populations. Samples were tested with licensed HIV-1/HIV-2 EIA test kits, FDA licensed HIV-1 Western Blot kits, and with the GS HIV-1 Western Blot. A total of 1430 sera/plasma samples (1115 serum; 315 plasma) and 345 dried blood spots (DBS) were tested by 7 independent clinical sites located in both high and low prevalence areas for HIV-1.

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### Reproducibility

The reproducibility of the GS HIV-1 Western Blot was evaluated at 7 sites with a panel of 6 specimens tested in duplicate on 5 runs on each of 3 lots. Each band was scored independently by 2 readers at each site. The percent of times each band was scored as present is shown in Table 1.

**Table 1: Precision**
**GS HIV-1 Western Blot**

| | % Frequency of Bands Present (number ≥ +/-) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Panel ID** | **gp160** | **gp120** | **p65** | **p55/51** | **gp41** | **p40** | **p31** | **p24** | **p18** |
| Negative Normal Donor | 0% | 0% | 0.5% | 0.5% | 0% | 0% | 0% | 0% | 0% |
| Negative Pooled Plasma | 0% | 0% | 1.0% | 1.0% | 0.5% | 0% | 1.4% | 2.4% | 1.0% |
| HIV-2 Positive Sample | 1.0% | 0% | 95.7% | 100% | 45.7% | 100% | 99.0% | 100% | 4.8% |
| Weak HIV-1 Positive Sample | 96.2% | 1.4% | 91.9% | 70.5% | 2.9% | 100% | 77.6% | 100% | 2.9% |
| HIV-1 High Positive Sample | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 99.5% |
| HIV-1 Low Positive Sample | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 93.8% |

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## SENSITIVITY AND SPECIFICITY

### Serum / Plasma

A study in low risk populations included 298 sera/plasma collected prospectively from normal blood donors, as well as 274 EIA repeatedly reactive sera/plasma selected retrospectively from blood center repositories (Table 2). This latter group of samples was chosen to include sera/plasma testing positive or indeterminate on a licensed HIV-1 Western Blot, as well as unselected with regard to reactivity on a licensed HIV-1 Western Blot test.

The GS HIV-1 Western Blot was positive for 63/63 (100%) blood center repository samples identified as positive by the licensed HIV-1 Western Blot, demonstrating comparable sensitivity. The GS HIV-1 Western Blot was negative for 68/78 (87.2%) samples found to be negative by the licensed HIV-1 Western Blot, demonstrating comparable specificity. (The GS HIV-1 Western Blot was indeterminate for the remaining 10 samples).

Of the 298 prospective normal donor samples, all were EIA non-reactive. The GS HIV-1 Western Blot was negative for 266 (89.3%) samples, indeterminate for 32 (10.7%) samples, and positive for none (0.0%). Twenty-six (26) of the 32 indeterminate samples were additionally tested with a licensed HIV-1 Western Blot, and found to be either indeterminate or negative (Table 2).

**Table 2: Evaluation of the GS HIV-1 Western Blot in Low Risk Populations (n=572)**

| Clinical Group | GS HIV-1 Western Blot Interpretation | Licensed Western Blot Interpretation (historical data) | | |
|---|---|---|---|---|
| | | **Positive** | **Indeterminate** | **Negative** |
| **Donors** EIA Repeatedly Reactive (Retrospective Study) (n=274) | **Positive (n=63)** | **63** | **0** | **0** |
| | Indeterminate (n=83[a]) | 0 | 73 | 10 |
| | Negative (n=128) | 0 | 60[b] | 68 |
| | | **Positive** | **Indeterminate** | **Negative** |
| **Donors** EIA Non-Reactive (Prospective Study) (n=298) | Positive (n=0) | 0 | 0 | 0 |
| | Indeterminate (n=32[c]) | 0[c] | 12[c] | 14[c] |
| | Negative (n=266[d]) | 0[d] | 4[d] | 17[d] |

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[a] One sample was originally entered into the study as Western blot positive (historical data) and found to be indeterminate on GS HIV-1 Western Blot. It was also indeterminate when retested with the licensed Western blot suggesting that the sample may have deteriorated during storage.

[b] These were repository specimens that had been stored frozen for various periods of time. Results of GS HIV-1 Western Blot are compared to historical licensed HIV-1 Western Blot data. No follow-up studies were done to determine if the observed differences were due to sample deterioration during storage.

[c] Six (6) of the samples that were indeterminate on the GS HIV-1 Western Blot were not tested on the licensed Western blot.

[d] Two hundred forty-five (245) of the 266 samples Negative on the GS HIV-1 Western blot were not tested on the licensed Western blot.

Additional studies in HIV high risk and AIDS populations included 525 samples collected prospectively and retrospectively (Table 3). Prospectively collected samples included 152 sera from AIDS patients; 20 sera

from ARC patients; 26 sera from HIV-1 asymptomatic patients; and 199 repository sera from high risk subjects. These included samples collected in a sequential manner from STD clinic patients (n = 49); homosexual males (n = 50); and hospital emergency room patients (n = 100) in a high prevalence area. Retrospective samples included 128 repository specimens testing EIA repeatedly reactive and positive on a licensed HIV-1 Western Blot. These samples were from various geographic locations including France (n = 12), Ghana (n = 5), Nairobi (n = 7), Nigeria (n = 12), Australia (n = 12), Thailand (n = 12), Zimbabwe (n = 12), Sierra Leone (n = 12), Mozambique (n = 2), Central African Republic (n = 14) and the USA (n = 28).

These studies demonstrated 172/172 (100%) positive results in AIDS and ARC populations and 176/176 (100%) positive results for high risk samples confirmed positive on a licensed HIV-1 Western Blot. Overall, there was 100% concordance of positive results between the GS HIV-1 Western Blot and the licensed Western blot. Of the 176 EIA non-reactive specimens from high risk populations, 8 were indeterminate and 137 were negative on both tests, demonstrating 82.4% concordance. There were 31 discordant results, all of which were indeterminate or negative on the two Western blot kits, as shown in Table 3.

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**Table 3: Comparison of the GS HIV-1 Western Blot with a Licensed HIV-1 Western Blot in AIDS, ARC, and High Risk Populations (n=525)**

| Clinical Group | GS HIV-1 Western Blot Interpretation | Licensed Western Blot Interpretation | | |
|---|---|---|---|---|
| | | Positive | Indeterminate | Negative |
| AIDS/ARC[a] EIA Repeatedly Reactive (n=172) | Positive (n=172) | 172 | 0 | 0 |
| | Indeterminate (n=0) | 0 | 0 | 0 |
| | Negative (n=0) | 0 | 0 | 0 |
| High Risk[b] EIA Repeatedly Reactive (n=177) | Positive (n=176) | 176 | 0 | 0 |
| | Indeterminate (n=1) | 0 | 1 | 0 |
| | Negative (n=0) | 0 | 0 | 0 |
| High Risk[c] EIA Non-Reactive (n=176) | Positive (n=0) | 0 | 0 | 0 |
| | Indeterminate (n=28) | 0 | 8 | 20 |
| | Negative (n=148) | 0 | 11 | 137 |

[a] AIDS (n =152); ARC (n = 20)

[b] HIV-1 Asymptomatic (n = 26); Prospective high risk (n = 23); HIV-1 confirmed positives from different geographic locations (n = 128)

[c] Prospective high risk (n = 176)

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Additional testing of GS HIV-1 Western Blot was performed on 333 specimens from persons with clinical conditions unrelated to HIV-1 that might result in a reactivity with proteins present. Table 4 summarizes these results. Although weak bands were occasionally present for viral proteins, none of the samples would meet the package insert requirements for an interpretation of POSITIVE.

**Table 4: GS HIV-1 Western Blot Results for Specimens from Patients with Conditions Unrelated to HIV-1 (n = 333)**

| | % Frequency of Bands Present (number ≥ + by one or both readers) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Clinical Condition | Number Studied | Number with Bands | gp160 | gp120 | p65 | p55/51 | gp41 | p40 | p31 | p24 | p18 |
| Toxoplasmosis | 20 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| Rheumatoid factor | 18 | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| ANA | 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| SLE | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Elevated IgG | 20 | 6 | 1* | 0 | 0 | 1 | 1* | 0 | 2 | 3* | 1 |
| Elevated IgM | 20 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 4 | 0 |
| Anti-HBV + | 20 | 2 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 2 | 0 |
| Anti-HTLV-I + | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Anti-HTLV-I/II + | 4 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| Anti-HTLV-II + | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Anti-CMV + | 19 | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| Anti-EBV + | 20 | 3 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| Anti-HAV IgM + | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Anti-HAV Total + | 10 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Anti-HCV + | 20 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| Anti-HSV + | 20 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| Anti-Rubella + | 19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cancer | 20 | 2 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 2 | 0 |
| Multi-Transfusion | 20 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| Multiparous women | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cirrhosis (type unknown) | 20 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |

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*None were interpreted as Western blot positive.

## Dried Blood Spots

In a comparative study to demonstrate equivalent performance of dried blood spots (DBS) compared to serum or plasma on the GS HIV-1 Western Blot, a total of 345 DBS paired to sera / plasma from low risk, high risk, and AIDS populations were tested at 5 clinical sites. The dried blood spots were tested with the licensed Genetic Systems™ HIV-1/HIV-2 EIA (DBS Procedure) and with the GS HIV-1 Western Blot. Results were com-

pared to the testing results of the paired sera/plasma on the GS HIV-1 Western Blot.

AIDS and high risk samples (Table 5) included a total of 248 paired sera and dried blood spot samples: 152 paired samples from AIDS patients; 20 paired samples from ARC patients; 26 paired samples from HIV-1 asymptomatic patients; and 50 simulated dried blood spots prepared with 50 prospective high risk sera. [Note: The simulated DBS were prepared by mixing equal volumes of serum and washed packed red blood cells (Type O, certified to be non-reactive for HIV and HBsAg)].

Paired dried blood spots demonstrated 172/172 (100%) positive results in AIDS and ARC populations and 26/26 (100%) positive results for HIV-1 asymptomatic patients. Of the 50 prospective high risk samples, 20 DBS and paired sera were EIA repeatedly reactive and 30 DBS and paired sera were EIA non-reactive. Twenty-seven (27) dried blood spots were negative, 3 were indeterminate and 20 were positive when tested with the GS HIV-1 Western Blot. By comparison, the 27 paired sera were negative, the 3 were indeterminate and the 20 were positive when tested with the GS HIV-1 Western Blot, demonstrating equivalence in sensitivity and specificity (concordance = 100%) for the testing of serum and dried blood spots.

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**Table 5: Comparison of Results for Paired Sera and Dried Blood Spots with GS HIV-1 Western Blot in AIDS, ARC, and High Risk Populations (n=248)**

| Clinical Group | Licensed HIV-1/HIV-2 EIA | GS HIV-1 Western Blot Interpretation | | |
|---|---|---|---|---|
| | | Positive | Indeterminate | Negative |
| **AIDS, ARC, High Risk Serum** | EIA Repeatedly Reactive (n=218) | 218 | 0 | 0 |
| | EIA Non-Reactive (n=30) | 0 | 3 | 27 |
| **AIDS, ARC, High Risk Dried Blood Spots** | EIA Repeatedly Reactive (n=218) | 218 | 0 | 0 |
| | EIA Non-Reactive (n=30) | 0 | 3 | 27 |

Low risk samples included 97 DBS paired to 97 sera / plasma from normal blood donors (Table 6). Of these, 96 DBS were EIA non-reactive and 1 was EIA repeatedly reactive. By comparison, none of the sera/plasma were EIA reactive. Sixty-three (63) DBS were negative, 34 were indeterminate and none were positive on the GS HIV-1 Western Blot. In testing the paired sera/plasma from these same normal donors, 78 were negative, 19 were indeterminate, and none were positive on the GS HIV-1 Western Blot [con-

cordance = (15 + 59)/97 = 76.3%] demonstrating comparable specificity for the testing of serum/plasma and dried blood spots.

**Table 6: Comparison of Results for Paired Sera/Plasma and Dried Blood Spots with GS HIV-1 Western Blot for Normal Blood Donors (n=97)**

| GS HIV-1 Western Blot Interpretation Serum or plasma | GS HIV-1 Western Blot Interpretation DBS | | |
|---|---|---|---|
| | Positive (n=0) | Indeterminate (n=34) | Negative (n=63) |
| Positive (n=0) | 0 | 0 | 0 |
| Indeterminate (n=19) | 0 | 15 | 4 |
| Negative (n=78) | 0 | 19 | 59 |

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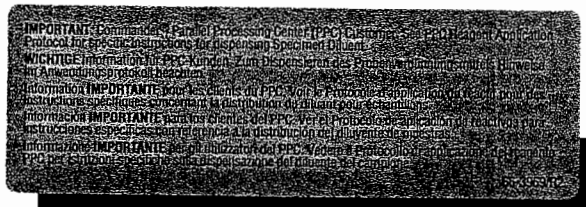
# ATTACHMENT 6

AIDS#37

# HUMAN IMMUNODEFICIENCY VIRUS TYPE 1
# HIVAB™ HIV-1 EIA

**NOTE CHANGES HIGHLIGHTED**


IMPORTANT: Commanders Parallel Processing Center (PPC) Customers
Protocol for specific instructions for dispensing Specimen Diluent.
WICHTIGE Information für PPC-Kunden: Zum Dispensieren des Probenverdünnungsmittels
Anwendungsprotokoll beachten.
Información IMPORTANTE: para los clientes del PPC: Vea el Protocolo de aplicación
instrucciones específicas empleando la distribución del diluyente para especímenes
Informação IMPORTANTE: para os clientes do PPC: Ver o Protocolo de aplicação
instruções específicas sobre a distribuição do diluente para amostras
Informazione IMPORTANTE per gli utilizzatori del PPC: Vedere il Protocollo di applicazione
PPC per istruzioni specifiche sulla dispensazione del diluente per campioni

## NAME AND INTENDED USE

**HIVAB™ HIV-1 EIA IS AN *IN VITRO* QUALITATIVE ENZYME IMMUNOASSAY FOR THE DETECTION OF ANTIBODY TO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) IN HUMAN SERUM OR PLASMA.**

66-8805/R5

ELISA Test

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January, 1997

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## HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ⊐HIVAB™ HIV-1 EIA

This product contains modifications to the ABBOTT HIV-1 EIA since January 16, 1987 which have improved sensitivity and includes the modification of March 27, 1989 that has made this product compatible with the COMMANDER® System. This product can be used with the Quantum™ II or Quantumatic™ without loss of sensitivity as an alternate procedure.

## NAME AND INTENDED USE

⊐HIVAB HIV-1 EIA is an *in vitro* qualitative Enzyme Immunoassay for the Detection of Antibody to Human Immunodeficiency Virus Type 1 (HIV-1) in Human Serum or Plasma.

## SUMMARY AND EXPLANATION OF THE TEST

Available data indicate that the acquired immunodeficiency syndrome (AIDS) is caused by a virus transmitted by sexual contact, exposure to blood (including sharing contaminated needles and syringes) or certain blood products, or transmitted from an infected mother to her fetus or child during the perinatal period.[1] Human Immunodeficiency Virus Type 1 (HIV-1) has been isolated from patients with AIDS and AIDS-related complex (ARC), and from healthy persons at high risk for AIDS.[2] The incidence of antibodies specific for HIV-1 in AIDS and ARC patients and persons at increased risk for AIDS is high. The prevalence of HIV-1 infection in people not known to be at increased risk is not known. The HIV-1 antibody enzyme-linked immunoassay (EIA) (the abbreviation "ELISA" is equally acceptable) was developed to detect antibodies to HIV-1 and was developed to identify potentially infectious units of donated blood and plasma. It has been established that repeatably reactive units of blood and plasma should be eliminated from the blood supply.[3]

In order to afford maximum protection of the blood supply, the EIA was designed to be extremely sensitive. As a result, non-specific reactions may be seen in samples from some people who, for example, due to prior pregnancy, blood transfusion, or other exposure, have antibodies to the human cells or media in which the HIV-1 is grown for manufacture of the EIA.[4] Because of these and other nonspecific reactions, it is appropriate to investigate specimens found to be reactive on EIA in a manner that gives improved predictability that HIV-1 antibody, in fact, is present. When a specimen reacts in an initial test (is initially reactive), the EIA should be repeated in duplicate on the same sample source. Reactivity in either or both of these duplicate tests (repeatably reactive) is highly predictive of the presence of antibody in people at increased risk for HIV-1 infection (e.g., homosexual men, hemophiliacs, or intravenous drug users). Repeatably reactive specimens obtained from people at increased risk for HIV-1 infection are usually found to contain antibodies by additional more specific, or supplemental, testing. However, when the EIA is used to screen populations in which the prevalence of HIV-1 infection is low (e.g., blood donors), nonspecific reactions may be more common (see Table II). Information about prevalence of HIV-1 infections in persons in various categories of risk, as well as clinical and public health guidelines, are available in the publication Morbidity and Mortality Weekly Reports.

Although for all clinical and public health applications of the EIA both the degree of risk for HIV-1 infection of the person studied and the degree of reactivity of the serum may be of value in interpreting the test, these correlations are imperfect. Therefore, in most settings it is appropriate to investigate repeatably reactive specimens by additional more specific, or supplemental tests.

## BIOLOGICAL PRINCIPLES OF THE PROCEDURE

The ⊐HIVAB HIV-1 EIA is manufactured from HIV-1 propagated in T-lymphocyte cell line H9/HTLV IIIB. The isolated virus is disrupted and inactivated with detergent and sonication. Two major viral proteins are purified from the lysate and then a combination of whole viral lysate and purified viral proteins is coated onto beads. Coated beads are incubated with a specimen diluent and human serum or plasma and appropriate controls. Any antibody to HIV-1 is bound to the HIV-1 antigens on the solid phase. After aspiration of the unbound material and washing of the bead, goat antibody to human IgG conjugated with horseradish peroxidase (Anti-Human IgG:HRPO) is incubated with the bead-antigen-antibody complex. Unbound enzyme conjugate is then aspirated and the beads washed. Next, o-Phenylenediamine (OPD) Solution containing hydrogen peroxide is added to the bead and, after incubation, a yellow-orange color develops in proportion to the amount of antibody to HIV-1 which is bound to the bead.

## REAGENTS

No. 3A11, ⊐HIVAB HIV-1 EIA KIT (100/1000 TESTS)

① 100/1000 HIV-1 Antigen Coated Beads. (Inactivated).

② 1 Vial (20 mL)/2 Vials (100 mL each) Anti-Human Conjugate (Goat). Anti-Human IgG (Goat): Peroxidase (Horseradish). Minimum Concentration: 0.01 µg/mL in TRIS Buffer. Preservatives: 0.01% Thimerosal and 0.015% Gentamicin.

③ 1 Vial (1.3 mL)/2 Vials (1.3 mL each) Positive Control. Inactivated Human Plasma Positive for Antibody to HIV-1. Minimum titer: 1:2. Preservative: 0.1% Sodium Azide.

④ 1 Vial (1.0 mL)/2 Vials (1.0 mL each) Negative Control. Human Plasma Negative for Anti-HIV-1, Anti-HCV and HBsAg. Preservative: 0.1% Sodium Azide.

⑤ 2 Vials (20 mL each)/5 Vials (100 mL each) Specimen Diluent containing 0.1% Triton X-100, Bovine and Goat Sera (minimum concentration of 5%) and Human T-Lymphocyte Lysate (minimum titer of 1:7500). Preservative: 0.1% Sodium Azide.

*

⑨ 1 Bottle (10 Tablets)/2 Bottles (40 Tablets each) OPD (o-Phenylenediamine•2HCl) Tablets. OPD/Tablet: 12.8 mg.

⑩ 1 Bottle (55 mL)/2 Bottles (220 mL each) Diluent for OPD (o-Phenylenediamine•2HCl). Citrate-Phosphate Buffer containing 0.02% Hydrogen Peroxide.

*There are no reagents 6, 7 and 8.

The stopping reagent is provided as an accessory to the ⊐HIVAB HIV-1 EIA Kit and consists of:

⑪ 1 *N* Sulfuric Acid, No. 7212 (Most U.S. and some International Locations).

Use of acid other than that supplied by ABBOTT may result in instability of the developed color. To be suitable as a stopping reagent, Sulfuric Acid must pass the following test each time it is prepared. The following test cannot be performed on the COMMANDER system. Use a Quantum II, Quantumatic, or suitable spectrophotometer to perform this test.

1. Pipette 300 µL of OPD Substrate Solution into 5 EIA reaction tubes or acid washed/distilled or deionized water rinsed tubes.
2. Add 2 mL of the 1 *N* Sulfuric Acid under test to each of the five tubes.
3. Measure the $A_{492}$ of the OPD/Acid Solution against distilled or deionized water at "0 TIME" and "120 MIN".
4. Calculate the Mean Absorbance at "0 TIME" and "120 MIN".
5. To be acceptable, acid must exhibit:
   a. an $A_{492}$ of less than 0.08 at "0 TIME" **and**
   b. a difference of less than 0.03 units in the values obtained at "0 TIME" and "120 MIN".

## WARNINGS AND PRECAUTIONS

**FOR *IN VITRO* DIAGNOSTIC USE**

**WARNING: FDA has licensed this test kit for use with human serum or plasma specimens only. Use of this licensed test kit with specimens other than those specifically approved for use with this test kit may result in inaccurate test results.**

- Handle all ⊐HIVAB HIV-1 EIA biological materials as though capable of transmitting infection.[10] HIV-1 antigen coated on beads has been inactivated by detergent and sonication prior to coating. Positive Control has been inactivated by heat treatment.
- The Negative and Positive Controls are provided in prediluted form. They should not be diluted as specimens.

### Safety Precautions

1. Do not pipette by mouth.
2. Do not smoke, eat, or drink in areas in which specimens or kit reagents are handled.
3. Wear disposable gloves while handling kit reagents or specimens and thoroughly wash hands afterward.
4. Avoid splashing or forming an aerosol.
5. Avoid contact of OPD and sulfuric acid with skin and mucous membranes. If these reagents come into contact with skin, wash thoroughly with water.
6. Dispose of all specimens and materials used to perform the test as if they contained infectious agents. The preferred method of disposal is autoclaving for a minimum of one hour at 121.5°C. Disposable materials may be incinerated. Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite.[11] Allow 30 minutes for decontamination to be completed.

**NOTE:** Liquid waste containing acid must be neutralized with a proportional amount of base prior to the addition of sodium hypochlorite.[12]

7. SPILLS: Spills should be wiped up thoroughly using either an iodophor disinfectant or sodium hypochlorite solution.

   Iodophor Disinfectant:

   - Iodophor disinfectant should be used at a dilution providing at least 100 ppm available iodine. Hy Sine, Ioprep, Mikroklene and Wescodyne are proprietary iodophors; the latter may be used at approximately a 1:100 to 1:150 final dilution in water. Neutralization of acid-containing spills is not required.[13-15]

   Sodium Hypochlorite:

   - Non acid-containing spills should be wiped up thoroughly with a 5% sodium hypochlorite solution.
   - Acid-containing spills should be wiped dry. Spill areas should then be wiped with a 5% sodium hypochlorite solution.

   Materials used to wipe up spills should be added to biohazardous waste matter for proper disposal.

### Handling Precautions

1. DO NOT MIX REAGENTS FROM DIFFERENT LOTS. Any OPD tablet, Diluent for OPD, or Sulfuric Acid lot, however, may be used with any ⊐HIVAB HIV-1 EIA kit lot.
2. Do not use kit beyond the expiration date.
3. DO NOT EXPOSE OPD REAGENTS TO STRONG LIGHT DURING INCUBATION OR STORAGE.
4. Avoid contact of the OPD Substrate Solution and 1 *N* Sulfuric Acid with any oxidizing agent. Do not allow Substrate Solution or 1 *N* Sulfuric Acid to come into contact with any metal parts. Prior to use, thoroughly rinse glassware used for OPD Solution with 1 *N* Sulfuric Acid using approximately 10% of the container volume. Follow with three washes of distilled water at the same volume.
5. Avoid microbial contamination of reagents when removing aliquots from the reagent vials. Use of disposable pipette tips is recommended.
6. USE A CLEAN DEDICATED DISPENSER FOR THE CONJUGATE SOLUTION TO AVOID NEUTRALIZATION.
7. Water for washing should be stored in clean containers to prevent contamination of water with HRPO inactivating substances.
8. If the desiccant obstructs the flow of beads, remove from bead bottle prior to dispensing beads. Replace desiccant in bottle and tightly cap bottle for storage. Do not store beads with dispenser attached to bottle.

## INSTRUCTIONS FOR PREPARATION OF OPD SUBSTRATE SOLUTION

The OPD (o-Phenylenediamine•2 HCl) Tablet must be dissolved in Diluent for OPD 5 to 10 minutes prior to use. The Solution must not be stored longer than 60 minutes before use.

Five to ten minutes prior to Color Development, prepare the OPD Substrate Solution as follows:

**NOTE:** 300 µL of OPD Substrate Solution is required for each test specimen as well as for two reagent blanks.

**CAUTION: Use pipettes and containers known to be metal free such as plastic ware or acid washed/distilled or deionized water rinsed glassware.**

1. Using a clean pipette, transfer into a suitable container 5 mL of Diluent for OPD for each tablet to be dissolved.
2. Transfer OPD Tablet(s) from bottle into container with Diluent for OPD using a nonmetallic forceps or equivalent. Return desiccant to bottle immediately, if removed to obtain a tablet, and close bottle tightly. Allow tablet(s) to dissolve. Do not use a tablet that is not intact.
3. Just prior to dispensing for the final incubation of the assay, swirl gently to obtain a homogeneous solution.

**OPD PREPARATION CHART**

| No. Tests | Tablets | Diluent |
|---|---|---|
| 13 | 1 | 5 mL |
| 28 | 2 | 10 mL |
| 43 | 3 | 15 mL |
| 58 | 4 | 20 mL |
| 73 | 5 | 25 mL |
| 88 | 6 | 30 mL |
| 103 | 7 | 35 mL |
| 118 | 8 | 40 mL |
| 133 | 9 | 45 mL |
| 148 | 10 | 50 mL |

## STORAGE INSTRUCTIONS

1. Store kit reagents at 2 to 8°C. OPD Tablets and 1 *N* Sulfuric Acid may be stored at 2 to 30°C.
2. Bring all reagents to room temperature (15 to 30°C) before use (approximately 30 minutes) and return to storage conditions indicated above immediately after use.

**CAUTION: Do not open OPD tablet bottle until it is at room temperature.**

3. Retain desiccant bags in OPD Tablet bottle at all times during storage.
4. Reconstituted OPD Solution MUST be stored at room temperature and MUST be used within 60 minutes. Do not expose to light.

## INDICATIONS OF INSTABILITY OR DETERIORATION OF REAGENTS

The OPD Substrate Solution (OPD plus Diluent for OPD) should be colorless to pale yellow. A yellow-orange color of the solution indicates that the reagent has been contaminated and must be discarded.

A value of less than 0.400 absorbance units for the difference between the Positive and Negative Control Means (P-N) may indicate technique errors or deterioration of the kit reagents or OPD reagents. Such runs should be repeated.

## SPECIMEN COLLECTION AND PREPARATION

HIVAB™ HIV-1 EIA may be performed on human serum or plasma.

1. If specimens are to be stored, they may be stored as serum or plasma at 2 to 8°C for up to 5 days. However, if storage periods greater than 5 days are anticipated, the specimens should be stored frozen. Avoid multiple freeze-thaw procedures. If specimens are to be shipped, they should be packed in compliance with federal regulations covering the transportation of etiologic agents.
2. Either serum or plasma may be used in the test. Remove the serum or plasma from the clot or red cells as soon as possible to avoid hemolysis. When possible, clear, nonhemolyzed specimens should be used. Specimens containing fibrin or any type of precipitate may give inconsistent test results. Such specimens should be clarified prior to assaying.

## PROCEDURE

**Materials Provided**

No. 3A11, HIVAB HIV-1 EIA Kit, 100/1000 Tests
(See REAGENTS for complete listing.)

The list of accessories required for the COMMANDER® System is found in the COMMANDER Manual. A combination of accessories is included with the COMMANDER System. The HIVAB HIV-1 EIA is designed to be compatible with the COMMANDER System; this product may also be used with a suitable spectrophotometer, Quantum™ II or Quantumatic.™

An optimum combination of the following accessories for Quantum II and Quantumatic is provided for performance of the tests ordered.

- Reaction Trays.
- Cover Seals (Tear along perforation for use with 20 well trays).
- Assay Tubes with Identifying Cartons (for transfer of beads from reaction trays).
- 1 *N* Sulfuric Acid, No. 7212 (Most U.S. and some International locations).

**Materials Required but not Provided (for Quantum II and Quantumatic)**

- Precision pipettes, EIA Pipetting Package (No. 7186), or similar equipment to deliver 200µL, 300µL and 1 mL.
- Precision pipette with disposable tip to deliver 10µL.
- Disposable, graduated pipettes or dispenser for measuring Diluent for OPD.
- Device for delivery of rinse solution such as a Gorman-Rupp™ Dispensing Pump, Heidolph Dispensing Pump (European customers only) or equivalent.
- An aspiration device for washing beads such as a Pentawash,® with a vacuum source such as a Gast® Vacuum Pump, or equivalent, and a double trap for retaining the aspirate and maintaining adequate vacuum, or use Qwikwash®.
- Water bath or equivalent capable of maintaining temperature at 39 to 41°C.
- Quantum Analyzer or spectrophotometer capable of reading absorbance at 492 nm.
- Single Bead Dispenser for dispensing one bead at a time from a 100 bead bottle.
- Multi-Bead Dispenser for dispensing twenty beads at a time from a 500 bead bottle.
- Nonmetallic forceps.
- Membrane Seal Puncture Tool for acid bottles.
- 1 *N* Sulfuric Acid.
- Predilution trays, test tubes and racks, or equivalent for predilution of samples.

*Included in EIA Pipetting Package

## ADDITIONAL REAGENTS AVAILABLE

1. Abbott OPD (o-Phenylenediamine•2 HCl) Reagent No. 6172.
2. 1 *N* Sulfuric Acid, No. 7212, (Most U.S. and some International Locations).

## ASSAY PROCEDURE

**Preliminary Comments**

Laboratories using the COMMANDER system should follow procedures in the COMMANDER Manual and the Reagent Applications Protocol as appropriate. The following assay procedure should be used with the Quantum II and Quantumatic.

## DILUTION OF SPECIMEN*

1a. Dispense 10 µL of each specimen into bottom of a predilution tray, test tube or equivalent. Add 200 µL of Specimen Diluent to each tube. Mix thoroughly. **DO NOT INCLUDE CONTROLS IN THIS STEP.**

1b. Dispense 10 µL of Diluted Specimen into bottom of appropriate wells of reaction tray.

1c. Dispense 200 µL of Specimen Diluent to each well containing 10µL diluted specimen.

## DILUTION OF CONTROLS*

2a. Dispense 10 µL of each control into bottom of appropriate wells of reaction tray (2 Negative and 3 Positive Controls).

2b. Dispense 200 µL of Specimen Diluent to each well containing a control.

## FIRST INCUBATION

3. Carefully add one bead to each well containing a control or diluted specimen.
4. Apply cover seal. Gently tap the tray to cover beads and remove any trapped air bubbles.
5. Incubate at 40°± 1°C for 1 hour ± 5 minutes.
6. Remove and discard cover seal. If a PENTAWASH® System is used, aspirate the liquid and wash each bead with 12 to 18 mL of distilled or deionized water. If an alternate system is used, consult your washing apparatus manual for appropriate washing procedures.

## SECOND INCUBATION

7. Pipette 200µL of Conjugate into each well containing a bead.
8. Apply new cover seal. Gently tap the tray to cover beads and remove any trapped air bubbles.
9. Incubate at 40° ± 1°C for 2 hours ± 10 minutes.
10. Remove and discard cover seal. Aspirate the liquid and wash each bead as in FIRST INCUBATION.

## COLOR DEVELOPMENT

11. Immediately transfer beads to properly identified assay tubes.
12. Pipette 300µL of freshly prepared OPD Substrate Solution into two empty tubes (Substrate Blanks) and then into each tube containing a bead.
    **NOTE:** Prime dispenser immediately prior to dispensing OPD Substrate Solution.
13. Cover and incubate at room temperature for 30 ± 2 minutes.
14. Add 1 mL of 1 *N* Sulfuric Acid to each tube.

## READING

15. Blank Spectrophotometer with a Substrate Blank at 492 nm.
16. Determine absorbance of Controls and Specimens at 492 nm.

*An automated procedure may be used to obtain an equivalent final dilution. Volume of diluted specimen and controls may vary from 200 to 420 µL without affecting the performance of the test.

## READING RESULTS

INSTRUMENTS: 1) Laboratories using the COMMANDER System should follow procedures in the COMMANDER manual and the HIV-1 Reagent Applications Protocol as appropriate. 2) Laboratories using Quantum II or Quantumatic should read absorbances as outlined below.

| Quantum II | Quantumatic |
|---|---|
| Use Mode 1.17 in Module A containing the HIVAB HIV-1 EIA protocol. | Use the following assay protocol for HIV-1<br>Cutoff Value = 1.000 ($NC\bar{x}$) + 0.100 ($PC\bar{x}$) + 0.000<br>P > N<br>No. of Neg. Controls = 2 |
| Line 10 of A Modules with List No. 4045-06 must be edited to read:<br>10: Max. Delta A = 1.999 | Min. Neg. Control = 0.010<br>Max. Neg. Control = 0.100<br>Neg. Aberrant Cutoff = 50.0%<br>No. of Pos. Control = 3<br>Min. Pos. Control = 0.400<br>Max. Pos. Control = 1.999<br>Pos. Aberrant Cutoff = 50.0%<br>Min. Control Diff. = 0.400<br>No. of Patient Replicates = 1 |

*A Modules with List Nos. 4045-01 through 4045-04 may be edited to run HIVAB HIV-1 EIA by contacting your ABBOTT Representative.

When using an edited procedure, the following checks must be manually performed:

1. Individual Negative Control values should be less than or equal to 0.100 and greater than or equal to 0.010.
2. Individual Negative Control values must be within the range 0.5 to 1.5 times the Negative Control Mean.
3. Individual Positive Control values should be less than or equal to 1.999 and greater than or equal to 0.400.
4. Individual Positive Control values must be within the range of 0.5 to 1.5 times the Positive Control Mean.

Refer to CALCULATION OF RESULTS for further detail.

For Instruments other than the Quantum: Following the reading of any specimen as outlined above in steps 1 through 4, cuvettes should be thoroughly rinsed with distilled or deionized water prior to reading the next tube. For calculations see RESULTS section.

## RESULTS

When a COMMANDER or Quantum Analyzer is used, refer to the appropriate Operator's Manual to determine which calculations are performed automatically. If a COMMANDER or Quantum Analyzer is not used, perform the following calculations on the assay data.

The presence or absence of antibody to HIV-1 is determined by relating the absorbance of the specimen to the Cutoff Value. The Cutoff Value is the absorbance of the Negative Control Mean plus 0.1 times the Positive Control Mean. For the run to be valid, the difference between the Means of the Positive and Negative Controls (P - N) should be 0.400 or greater. If not, technique may be suspect and the run must be repeated. If the P - N Value is consistently low, deterioration of reagents may be suspect.

### Calculation of Results

1. Calculation of Negative Control Mean Absorbance ($NC\bar{x}$).
   Determine the Mean of the Negative Control Values.

   Example:

| Negative Control Sample No. | Absorbance |
|---|---|
| 1 | 0.060 |
| 2 | 0.056 |
| TOTAL | 0.116 |

$$\frac{\text{Total Absorbance}}{2} = \frac{0.116}{2} = 0.058\ (NC\bar{x})$$

Individual Negative Control Values must be less than or equal to 0.100 and greater than or equal to 0.010.

Individual Negative Control Values must be within the range 0.5 to 1.5 times the Negative Control Mean. If one value is outside this range, the test must be repeated. If more than an occasional value falls outside this range, technique problems should be investigated.

2. Calculation of Positive Control Mean Absorbance ($\overline{PC}$).
Determine the Mean of the Positive Control Values.

Example:

| Positive Control Sample No. | Absorbance |
|---|---|
| 1 | 0.835 |
| 2 | 0.925 |
| 3 | 1.015 |
| TOTAL | 2.775 |

$$\frac{\text{Total Absorbance}}{3} = \frac{2.775}{3} = 0.925\ (\overline{PC})$$

Individual Positive Control Values must be less than or equal to 1.999 and greater than or equal to 0.400.

Individual Positive Control Values must be within the range 0.5 to 1.5 times the Positive Control Mean. If one value is outside the acceptable range, discard this value and recalculate the Mean. If two values are outside the range, the test should be repeated.

3. Calculation of the Cutoff Value
Cutoff Value = $\overline{NC} + (0.1 \times \overline{PC})$
Example:
$\overline{NC} = 0.058$
$\overline{PC} = 0.925$
Cutoff Value = $0.058 + (0.1 \times 0.925) = 0.151$

4. Calculations for Determining P - N
Example:
$\overline{NC} = 0.058$
$\overline{PC} = 0.925$
P - N = (0.925 - 0.058) = 0.867
For the run to be valid, the P - N Value should be 0.400 or greater. If not, technique or deterioration of reagents may be suspect and the run should be repeated.

## INTERPRETATION OF RESULTS

1. Specimens with absorbance values less than the cutoff value are considered not reactive by the criteria of ⊐HIVAB™ HIV-1 EIA and may be considered negative for the antibody. Further testing is not required.
2. Specimens with absorbance values equal to or greater than the cutoff value are considered reactive (initially reactive) by the criteria of ⊐HIVAB HIV-1 EIA, but before interpretation, the original sample source should be retested in duplicate. If either duplicate retest is reactive, the specimen is considered repeatably reactive.
3. Initially reactive specimens which do not react in either of the duplicate repeat tests are considered negative for antibodies to HIV-1.
4. If the specimen is repeatably reactive, the probability that antibodies to HIV-1 are present is high, especially in specimens obtained from subjects at increased risk for HIV-1 infection or in specimens with very high absorbance values.[5] In most settings it is appropriate to investigate repeatably reactive specimens by additional more specific, or supplemental tests. Specimens found repeatably reactive by EIA and positive by additional more specific, or supplemental testing are considered positive for antibodies to HIV-1. The interpretation of results of specimens found repeatably reactive by EIA and negative on additional more specific testing is unclear; further clarification may be obtained by testing another specimen taken three to six months later.

## LIMITATIONS OF THE PROCEDURE

The ⊐HIVAB HIV-1 EIA antibody procedure and the Interpretation of Results must be followed closely when testing for the presence of antibodies to HIV-1 in plasma or serum from individual subjects. Because the EIA was designed to test individual units of blood or plasma, most data regarding its interpretation were derived from testing individual samples. Insufficient data are available to interpret tests performed on other body specimens, pooled blood or processed plasma, and products made from such pools; testing of these specimens is not recommended.

⊐HIVAB HIV-1 EIA detects antibodies to HIV-1 in blood and thus is useful in screening blood and plasma donated for transfusion and further manufacture, in evaluating patients with signs or symptoms of AIDS, and in establishing prior infection with HIV-1. Clinical studies continue to clarify and refine the interpretation and medical significance of the presence of antibodies to HIV-1.[6] For most uses it is recommended that repeatably reactive specimens be investigated by an additional more specific, or supplemental test. A person who has antibodies to HIV-1 is presumed to be infected with the virus, except that a person who has participated in an HIV vaccine study may develop antibodies to the vaccine and may or may not be infected with HIV. Clinical correlation is indicated with appropriate counseling, medical evaluation and possibly additional testing to decide whether a diagnosis of HIV infection is accurate. Such an evaluation should be considered an important part of HIV-1 antibody testing and should include test result confirmation on a freshly drawn sample.

AIDS and AIDS-related conditions are clinical syndromes and their diagnosis can only be established clinically.[7] EIA testing alone cannot be used to diagnose AIDS, even if the recommended investigation of reactive specimens suggests a high probability that the antibody to HIV-1 is present. A negative test result at any point in the investigation of individual subjects does not preclude the possibility of exposure to or infection with HIV-1. The risk of an asymptomatic person with a repeatably reactive serum sample developing AIDS or an AIDS-related condition is not known.[8]

Data obtained from testing persons both at increased and at low risk for HIV-1 infection suggest that repeatably reactive specimens with high absorbance on EIA are more likely to demonstrate the presence of the HIV-1 antibodies by additional more specific, or supplemental testing.[9] Reactivity at or only slightly above the cut-off value is more frequently nonspecific, especially in samples obtained from persons at low risk for HIV-1 infection; however, the presence of antibodies in some of these specimens can be demonstrated by additional more specific, or supplemental testing.

## EXPECTED RESULTS

### A. SPECIFIC PERFORMANCE CHARACTERISTICS

### Precision

Assay reproducibility was determined by assaying five specimens in replicates of three on four consecutive runs over a period of four days using the same lot of material at three sites.

The Intra-assay and Inter-assay Standard Deviation (SD) and Coefficient of Variation (%CV) were calculated (Table I). Mean S/CO is defined as the Mean Sample Absorbance divided by the Cutoff. S/CO ratios greater than or equal to 1.00 are Reactive, and S/CO ratios less than 1.00 are Negative. Mean absorbance is defined as the Mean of all controls tested.

**TABLE I**
**⊐HIVAB HIV-1 EIA Reproducibility**

| Specimen | Mean S/CO | Intra-assay SD | Intra-assay % CV | Inter-assay SD | Inter-assay % CV |
|---|---|---|---|---|---|
| 1 | 0.52 | 0.060 | 11.6 | 0.075 | 14.4 |
| 2 | 1.81 | 0.150 | 8.3 | 0.185 | 10.2 |
| 3 | 3.43 | 0.256 | 7.5 | 0.289 | 8.4 |
| 4 | 5.80 | 0.459 | 7.9 | 0.617 | 10.6 |
| 5 | 9.79 | 0.802 | 8.2 | 0.863 | 8.8 |

| Control | Mean Absorbance | Intra-assay SD | Intra-assay % CV | Inter-assay SD | Inter-assay % CV |
|---|---|---|---|---|---|
| Negative | 0.037 | 0.0080 | 21.6 | 0.0080 | 21.6 |
| Positive | 1.250 | 0.0643 | 5.1 | 0.1315 | 10.5 |

### Sensitivity and Specificity

At present there is no recognized standard for establishing the presence and absence of HIV-1 antibody in human blood. Therefore sensitivity was computed based on the clinical diagnosis of AIDS and specificity based on random donors.

All data in this package insert were obtained with the COMMANDER® System.

**The ABBOTT studies show that:**

1. Sensitivity based on an assumed 100% prevalence of HIV-1 antibody in AIDS patients is estimated to be 100% (144 patients tested).
2. Specificity based on an assumed zero prevalence of HIV-1 antibody in random donors is estimated to be 99.9%* (4777 random donors tested).

*In these calculations, three samples of the eight total repeatably reactive specimens that confirmed have been excluded.

### B. REACTIVITY IN RANDOM DONOR POPULATIONS

The ability of ⊐HIVAB HIV-1 EIA to detect antibody to HIV-1 in blood bank donor specimens is shown in Table II. The data include 4777 serum and plasma samples obtained from blood donors at three geographically distinct blood banks. The number of specimens found repeatably reactive for antibody to HIV-1 by ⊐HIVAB HIV-1 EIA with testing performed in the automated mode using the COMMANDER System was 0.17%. The percent of repeatably reactive specimens obtained from blood donors at three geographically distinct blood and plasma centers was 0.00 to 0.30%.

**TABLE II**
**Detection of Antibody to HIV-1 in Plasma (P) Samples (Sites 1 and 3) and Serum (S) Samples (Sites 2 and 3) from Blood and Plasma Donors.**

| Site | Number Tested | ⊐HIVAB HIV-1 EIA Nonreactive | Initially Reactive | Repeatably Reactive |
|---|---|---|---|---|
| 1(P) | 1048 | 1047 (99.90%) | 1 (0.10%) | 0 (0.00%) |
| 2(S) | 993 | 990 (99.70%) | 3 (0.30%) | 3* (0.30%) |
| 3(P) | 1679 | 1674 (99.70%) | 5 (0.30%) | 5** (0.30%) |
| 3(S) | 1057 | 1055 (99.81%) | 2 (0.19%) | 0 (0.00%) |
| **Total** | **4777** | **4766 (99.77%)** | **11 (0.23%)** | **8 (0.17%)** |

*In these studies, the three repeatably reactive samples were indeterminate on the FDA licensed western blot.

**In these studies, three of five repeatably reactive samples were positive on the FDA licensed western blot. Two of five repeatably reactive samples were indeterminate on the FDA licensed western blot.

### C. REACTIVITY IN PATIENT POPULATIONS

The reactivity of ⊐HIVAB HIV-1 EIA was determined by testing serum and plasma specimens from patients diagnosed as having AIDS, AIDS-related complex (including patients with lymphadenopathy and idiopathic thrombocytopenia purpura) and high risk groups. (See Table III).

**TABLE III**
**Detection of Antibody to HIV-1 in Serum and Plasma Samples from Patients with AIDS, AIDS-Related Complex (ARC) and High Risk Groups**

| Group | No. of Specimens Tested | ⊐HIVAB HIV-1 EIA COMMANDER System No. Reactive | ⊐HIVAB HIV-1 EIA COMMANDER System (% Reactive) |
|---|---|---|---|
| AIDS | 144 | 144 | (100%) |
| ARC | 84 | 84 | (100%) |
| *High Risk Groups | 150 | 150 | (100%) |

*Previously tested and found repeatably reactive by ⊐HIVAB HIV-1 EIA (List 3036 and 2A95) and positive by the FDA licensed western blot.

A total of 50 samples from patients with medical conditions unrelated to AIDS were tested at two centers. None of the samples was reactive. Medical conditions including 25 positive RPR patients, 9 with other viral infections, 1 with toxoplasmosis, 3 with systemic lupus erythematosus, 3 with rheumatoid arthritis, 7 with hypergammaglobulinemia, and 2 with autoimmune conditions.

### D. REACTIVITY OF SEROCONVERSION DONORS

Evidence for improved sensitivity of the current kit (List 3A11) was obtained from studies of seroconversion sera tested at Abbott Laboratories (See Table IV). Ten seroconversion series, comprising 56 samples, were obtained from donors who were plasmapheresed regularly. No risk factors are known. All the samples tested by FDA licensed western blot were negative or indeterminate. None of the eight series had a band at p31; however all series showed other bands characteristic of an HIV-1 infection. These donors (with the exception of 72593, C1066 and H831) have been described previously.[16] Each sample was tested in triplicate in two separate assays on two separate days. The current kit (List 3A11) run on the COMMANDER System detected the presence of HIV-1 antibody earlier than the previously FDA licensed ABBOTT HIV-1 EIA (List 2A95).

**TABLE IV**
**Performance on Seroconversion Sera of the Current ☐HIVAB™ HIV-1 EIA (List 3A11) Compared with the ABBOTT HIV-1 EIA Kit (List 2A95) Prior to Modification and the FDA licensed western blot**

| Donor/(No.) | | Days of Donation | Replicates Reactive* HIV-1 EIA (List 2A95) COMMANDER | HIV-1 EIA (List 3A11) COMMANDER | Bands Found on FDA Licensed Western Blot |
|---|---|---|---|---|---|
| 3988 | (1) | 1 | 0/3 | 0/3 | None |
| | (2) | 3 | 0/3 | 0/3 | None |
| | (3) | 8 | 0/3 | 0/3 | 24 |
| | (4) | 10 | 0/3 | 0/3 | 24 |
| | (5) | 17 | 3/3 | 3/3 | 24,41,160 |
| | (6) | 20 | 3/3 | 3/3 | 24,41,160 |
| 6108 | (1) | 1 | 0/3 | 3/3 | 24 |
| | (2) | 5 | 0/3 | 3/3 | 24,55 |
| | (3) | 9 | 1/3 | 3/3 | 17,24,55 |
| | (4) | 12 | 1/3 | 3/3 | 17,24,41,55 |
| | (5) | 16 | 3/3 | 3/3 | 17,24,41,55 120,160 |
| | (6) | 19 | 3/3 | 3/3 | 17,24,41,55,66 120,160 |
| | (7) | 23 | 3/3 | 3/3 | 17,24,41,51,55 66,120,160 |
| | (8) | 26 | 3/3 | 3/3 | 17,24,41,51,55, 66,120,160 |
| 6977 | (1) | 1 | 3/3 | 3/3 | 24 |
| | (2) | 6 | 3/3 | 3/3 | 24 |
| | (3) | 10 | 3/3 | 3/3 | 24,41,160 |
| | (4) | 16 | 3/3 | 3/3 | 24,41,120,160 |
| | (5) | 20 | 3/3 | 3/3 | 24,41,120,160 |
| 13078 | (1) | 1 | 0/3 | 0/3 | None |
| | (2) | 6 | 0/3 | 0/3 | None |
| | (3) | 8 | 0/3 | 2/3 | None |
| | (4) | 14 | 3/3 | 3/3 | 24 |
| | (5) | 17 | 3/3 | 3/3 | 24,41,55 |
| | (6) | 21 | 3/3 | 3/3 | 24,41,55 |
| | (7) | 24 | 3/3 | 3/3 | 24,41,55 |
| 46320 | (1) | 5 | 3/3 | 3/3 | 17,24,51,66 120,160 |
| | (2) | 9 | 3/3 | 3/3 | 17,24,51,66 120,160 |
| 46402 | (1) | 1 | 0/3 | 2/3 | 24 |
| | (2) | 8 | 0/3 | 3/3 | 24,160 |
| | (3) | 10 | 3/3 | 3/3 | 24,160 |
| | (4) | 17 | 3/3 | 3/3 | 24,51,66,160 |
| | (5) | 22 | 3/3 | 3/3 | 24,51,66,160 |
| 103524 | (1) | 1 | 1/3 | 3/3 | 24,41,66 |
| | (2) | 5 | 3/3 | 3/3 | 24,41,51,55,66 |
| | (3) | 8 | 3/3 | 3/3 | 24,41,51,55,66 |
| 72593 | (1) | 1 | 3/3 | 3/3 | 24 |
| | (2) | 3 | 1/3 | 3/3 | 24 |
| | (3) | 10 | 3/3 | 3/3 | 24,120,160 |
| | (4) | 16 | 3/3 | 3/3 | 24,120,160 |
| | (5) | 22 | 3/3 | 3/3 | 24,120,160 |
| | (6) | 24 | 3/3 | 3/3 | 24,120,160 |
| C1066 | (1) | 1 | 0/3 | 0/3 | None |
| | (2) | 5 | 0/3 | 0/3 | None |
| | (3) | 16 | 1/3 | 3/3 | 24,120,160 |
| | (4) | 21 | 2/3 | 3/3 | 24,120,160 |
| | (5) | 23 | 3/3 | 3/3 | 24,120,160 |
| | (6) | 28 | 3/3 | 3/3 | 24,120,160 |
| | (7) | 30 | 3/3 | 3/3 | 24,120,160 |
| H831 | (1) | 1 | 0/3 | 0/3 | None |
| | (2) | 4 | 0/3 | 0/3 | None |
| | (3) | 15 | 0/3 | 0/3 | None |
| | (4) | 22 | 0/3 | 0/3 | None |
| | (5) | 52 | 0/3 | 2/3 | 24,55,120 |
| | (6) | 56 | 0/3 | 3/3 | 24,55,120 |
| | (7) | 60 | 0/3 | 3/3 | 24,55,120 |
| Overall Percent Detected of 56 Samples | | | 58 | 76 | |

*A total of 3 replicates per sample were tested, table entries represent number of replicates reactive/number of replicates tested.

# ATTACHMENT 7

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# An Evaluation of the Polymerase Chain Reaction in HIV-1 Seronegative Men

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**Summary:** The apparent detection of human immunodeficiency virus (HIV-1) DNA by the polymerase chain reaction (PCR) in seronegative individuals has been the subject of great concern. In this study, 324 seronegative participants in the San Francisco Men's Health Study were evaluated for evidence of infection using a PCR testing algorithm with multiple amplifications targeting different regions of the HIV-1 genome. While most PCR reactions were negative, 8.6% of the specimens showed weak reactivity with one or more primer sets. However, all were negative with at least one primer set and no definitively positive specimens were identified. This study addressed the possibility that some of these PCR reactions might represent latent infection or abortive exposure, leaving residual integrated DNA, rather than false-positive reactions. The frequency of such reactions was determined in homosexual men who have been at risk for HIV-1 infection and in exclusively heterosexual men who have little or no past exposure. The results demonstrate an identical frequency and distribution of equivocal PCR reactions in both populations. Assuming that there is minimal HIV-1 infection among seronegative heterosexual men in San Francisco, we conclude that PCR testing does not provide evidence for a reservoir of occult HIV-1 infection in seronegative homosexual men. **Key Words:** Human immunodeficiency virus—Occult infection—Polymerase chain reaction.

The polymerase chain reaction (PCR) for the in vitro amplification of DNA is an extremely sensitive method for detecting genomic sequences of infectious agents (1), including the human immunodeficiency virus, type 1 (HIV-1) (2). The reported detection by PCR of HIV-1 DNA in individuals at risk of infection who have negative results by serological testing has led to concern that there may be a substantial reservoir of occult infection (3–6). Given the potential ramifications of these reports and the failure of several studies to replicate these findings (7–11), the epidemiologic significance of positive PCRs in seronegative individuals remains controversial. A major weakness in many studies of PCR has been the failure to report the rate of positive PCRs in concurrently tested seronegative individuals without HIV-1 risk factors.

Since PCR is presumed to be more sensitive than serologic testing, there is no "gold standard" laboratory test that defines the true infection status, and a true positive PCR test cannot be distinguished from a false positive. One solution to this problem is to perform the assay in matched populations with

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and without risk factors for the infection and compare the frequency of positive reactions.

We have performed such an analysis in the San Francisco Men's Health Study (SFMHS), a population-based sample of men who, in 1984, were 24–55 years of age and living in 19 contiguous census tracts of San Francisco (12). This cohort was originally composed of 396 seropositive and 410 seronegative self-reported homosexual or bisexual men and 204 exclusively heterosexual controls.

Among the 410 homosexual men who had seronegative results at entry into the cohort, 42 (10%) have seroconverted in the succeeding 5 years and there has been a stable seroconversion rate of about 1% per year for the past 3 years. Among the 204 seronegative heterosexual men, only one (0.5%) seroconverted in the same period. For the purpose of this study, we have made the assumption that the cumulative risk for exposure to HIV-1 has been negligible in the heterosexual controls compared with the risk in the homosexual and bisexual men.

## METHODS

### Samples

Fresh whole blood samples were collected prospectively from participants in the SFMHS from April 1989 through April 1990. A total of 375 seronegative participants visited the clinic during this period and 364 provided one or more whole blood specimens for PCR testing. Men who either declined to state a sexual preference or whose self-reported orientation changed during the 4 years of follow-up observation were also excluded from this analysis (n = 40). The resulting study population consisted of 324 individuals, 105 exclusively heterosexual men and 219 homosexual or bisexual men, all of whom had negative results for HIV-1 antibody. Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation through Ficoll/diatrizoate (LSM, Organon Teknika, Durham, NC, U.S.A.) and frozen in aliquots of $1 \times 10^6$ PBMCs per vial in a solution of 90% fetal bovine serum and 10% dimethyl sulfoxide. Subsequently, the frozen PBMCs were thawed and subjected to PCR testing as described below.

### PCR Procedures

The PCR testing methods used in these studies were similar to published methods for the amplification and detection of HIV-1 DNA (13). Briefly, $10^6$ frozen PBMCs were thawed, washed once, and resuspended in 0.5 ml of lysis buffer consisting of 50 m*M* KCl, 1 m*M* $MgCl_2$, 10 m*M* Tris-Cl pH 8.3, 0.5% NP-40, 0.5% Tween-20, and 60 μg/ml of proteinase K. These samples were incubated at 60°C for 60 min to release target DNA and then inactivated by incubation at 95–100°C for 10 min. A portion of each lysate equivalent to $10^5$ PBMCs was mixed with synthetic DNA primers, heat-stable DNA polymerase, and deoxynucleotides. The target sequences were then amplified by temperature cycling. A portion of the amplified product and a labeled synthetic oligonucleotide probe, complementary to the amplified sequence, were mixed in an appropriate hybridization solution and incubated at 55°C for 10–30 min. The hybridized material was separated by electrophoresis through a 10% polyacrylamide gel and the HIV-1-specific hybrid was identified by autoradiography. A separate amplification of HLA-DQ-α was also performed on each specimen to verify the presence of adequate amplifiable DNA and was evaluated by electrophoresis in an agarose gel and visualization by ethidium bromide staining. All aliquots for which HIV-1 results were tabulated were positive for HLA-DQ-α.

Individual PCR reactions were assigned a visual intensity score on a scale of 0–4 that is illustrated in Fig. 1. For this analysis, samples with an intensity score of 1 were considered to be "weak positives." Control experiments using mixtures of infected and uninfected H9 cells indicate that this corresponds to about one infected cell in a background of $10^6$ uninfected cells. Scores of 2 and 3 were grouped as "moderate," and scores of 4 were classified as "strong positives." In our experience, seropositive SFMHS participants generally have intensity

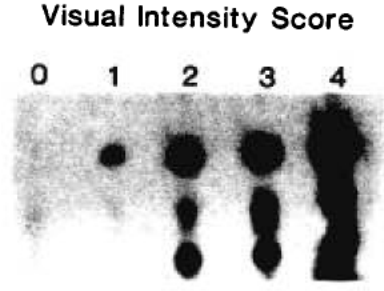


**FIG. 1.** Visual intensity scoring for five representative PCR reactions. Detection HIV-1 was performed using DNA lysates equivalent to $1 \times 10^5$ patient PBMCs that were; amplified with a *gag* primer pair (SK38/39), hybridized with a $^{32}P$-labeled oligonucleotide probe (SK19), separated on a 10% polyacrylamide gel, and visualized by autoradiography.

scores of 2–4 and most of the reactions with intensity scores of 1 are observed in specimens from seronegative individuals and are negative on repeat testing with alternate primer sets (data not shown).

### Testing Algorithm

Samples were subjected to a testing algorithm that involved the use of multiple amplification reactions as necessary, targeting up to three different regions of the HIV-1 genome for each specimen. Primer pairs used in these studies included two from the HIV-1 *gag* gene (SK38/39 and SK101/145) and one *env* gene (SK68/69). The sequences of these primer sets and their sensitivity for North American isolates of HIV-1 have been described elsewhere (2,9,13,14). A similar test algorithm has been previously validated in the context of a blinded multicenter proficiency trial of PCR in which this laboratory achieved an overall sensitivity and specificity of 98.0 and 97.1%, respectively. Of the 99 specimens from seropositive individuals, one had "weak positive" signals with both *gag* primer sets, a negative score with *env* primers, and was classified as indeterminate. One specimen was a strong positive but had been inadvertently switched with an adjacent negative specimen, resulting in one false negative and one false positive. The remaining 97 samples had moderate or strong signal intensities.

The complete diagnostic testing algorithm consists of amplification with two different primer pairs (one *gag* and one *env* pair or two *gag* pairs). Specimens were reported as positive if both reactions have intensity scores of 2–4 and as negative if both reactions have a score of 0. Reactions with intensity scores of 1 are considered discordant with both negative and positive designations. If the two results are discordant, an additional amplification is performed using the third primer pair. Where two of three of the reactions agree, a final result is reported. Specimens that remain discordant after three reactions are reported as indeterminate.

In previous studies, it was determined that the SK38/39 primer pair had extremely high sensitivity in this patient population (about 99%). For the purpose of this study, the algorithm was modified so that specimens that were negative after amplification with SK38/39 alone were designated negative and included in the analysis. Approximately half of the specimens were subjected to the full test algorithm irrespective of their SK38/39 result.

## RESULTS

Testing for the presence of HIV-1 genomic sequences was performed on PBMC specimens from 324 seronegative SFMHS participants, 219 homosexual/bisexual men and 105 exclusively heterosexual men. Each specimen was subjected to the PCR testing as described earlier. The number of specimens that showed discrepancies between the first two primer pairs and the final interpretation of HIV-1 infection status after subsequent repeat testing are shown in Table 1. The frequency of specimens requiring third primer pair testing was identical in both risk groups (8.6%). No positive specimens were identified and only two specimens remained indeterminate after completion of the testing algorithm, one from a homosexual and one from a heterosexual.

The number and frequency of all nonnegative PCRs in the two risk groups as well as the distribution of intensity scores are shown in Table 2. Of 551 PCRs performed, a total of 43 (7.8%) nonnegative reactions were observed in 28 of 324 (8.6%) of the specimens. Nonnegative reactions were observed in 29 of 380 (7.6%) of the PCR reactions performed on samples from homosexual/bisexual men, and in 14 of 171 (8.2%) of the reactions performed on samples from exclusively heterosexual men.

Nonnegative reactions occurred with all three primer sets, SK38/39, SK101/145, and SK68/69, at rates of 6.3, 14.7, and 4.4%, respectively, and the relative proportions were similar in both risk groups. While HIV-1 sequence variation can affect

**TABLE 1.** *Overall interpretation of PCR testing*

| Group | No. of patients | Initial discrepant, no. (%) | No. positive | Final interpretation | |
|---|---|---|---|---|---|
| | | | | No. negative | No. indeterminate (%) |
| Homosexual | 219 | 19 (8.7) | 0 | 218 | 1 (0.5) |
| Heterosexual | 105 | 9 (8.6) | 0 | 104 | 1 (1.0) |
| Total | 324 | 28 (8.6) | 0 | 322 | 2 (0.6) |

TABLE 2. *Distribution of individual PCRs*

| Group | Total reactions | Negative | Positive reactions | | | Nonnegative, no. (%) |
|---|---|---|---|---|---|---|
| | | | Weak | Moderate | Strong | |
| Homosexual | 380 | 351 | 21 | 7 | 1 | 29 (7.6) |
| Heterosexual | 171 | 157 | 9 | 5 | 0 | 14 (8.2) |
| Total | 551 | 508 | 30 | 12 | 1 | 43 (7.8) |

the relative performance of PCR primers, our experience indicates that large fluctuations in the specificity of a primer set also occur in proportion to the volume of PCR testing performed with that primer set. During a previous study (14), most of the nonnegative reactions in seronegative individuals were attributable to SK38/39, reflecting the heavy use of this primer set and increasing the potential for the contamination of negative samples with previously amplified product.

Finally, most of the nonnegative reactions had the lowest relative intensity, including 21 of 29 (72%) from homosexuals and nine of 14 (64%) from heterosexuals. Only one reaction had an intensity score of 4 (Table 2). This reaction occurred with one of the *gag* region primer sets (SK38/39) and was negative using the alternate *gag* primers (SK101/145) and the *env* region primer set (SK68/69).

## DISCUSSION

Since the first reports of positive PCRs in seronegative individuals (3,4), there has been considerable concern that there might be a substantial reservoir of occult HIV-1 infection with the potential for additional transmission that would not be prevented by present testing and counseling strategies. Since that time there have been several reports describing the propensity for false-positive results inherent in PCR testing (15,16), and several attempts have been made to establish the frequency of HIV-1 infection in seronegative populations with risk factors for infection (7–11). A major deficiency in many of these studies has been the absence of sufficient concurrent "true" negative controls to establish the background false-positive rate. Another difficulty with many of these studies is the failure to use other confirmatory methods such as antigen detection or HIV isolation since the PCR is assumed to be inherently more sensitive. In one report (3), isolation of HIV was used to confirm PCRs but the culture and PCR were not performed on the same specimen.

Finally, longitudinal studies of seronegative individuals who eventually seroconverted showed only sporadic PCR reactivity with generally weak signal intensity (4). We have suggested that if this is not a laboratory artifact, it might be evidence for some kind of abortive exposure resulting in DNA integration but insufficient viral replication to induce antibody formation (17).

In this report we have taken a statistical approach in which the frequency of nonnegative PCR reactions has been compared in populations with different levels of risk for exposure to HIV-1. If such sporadic PCR reactions represent true biologic phenomena, one would expect to detect a difference between groups with substantially different histories of exposure to HIV-1. The results presented here demonstrate that there is an identical frequency and distribution of nonnegative reactions (about 8% of all reactions) in seronegative populations with extremely different historical risk for exposure to HIV-1. We conclude that stable occult or latent HIV-1 infection in seronegative homosexual men, if it exists at all, is an extremely rare phenomenon that is of little or no epidemiologic significance.

It is of interest to note that, in a previous study of seronegative regularly repeating blood donors (14), we observed a similar rate of indeterminate specimens (one of 105) and a somewhat higher rate of nonnegative PCRs (38 of 246, or 15%). These results illustrate the presence and variability of false reactivity, inherent in current PCR methods, and argue for the simultaneous performance of many negative controls to monitor changing false-positive rates.

This study does not directly address the possibility that, in some cases, weak and sporadic PCR positivity might be present during a long incubation period between infection with HIV-1 and the first appearance of specific antibody. The SFMHS co-

hort has had a stable seroconversion rate of 1% per year for the past 3 years. Therefore, an incubation period as long as 2 years would result in only about 10 individuals in this population incubating virus during the study period. Weak and sporadic PCRs in these individuals might not be detectable over a background rate of almost 9%. However, the failure to identify any definitively positive specimens is consistent with the conclusion that PCR does not detect a significant window period between infection and seroconversion. Finally, none of the 28 individuals in this study with nonnegative PCR reaction have seroconverted during the subsequent 12 months of follow-up observation. A definitive answer to the seroconversion question will require a careful retrospective analysis of seroconvertors, which is presently under way using blinded testing in two laboratories, a large number of "true-negative" controls, and established PCR testing algorithms.

**Acknowledgment:** We gratefully acknowledge the expert technical assistance of Eileen Liu and Ruth Cox. This work was supported by NIH/NIAID contract no. N01-AI-82515 through a subcontract to the California Public Health Foundation.

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# ATTACHMENT 8

# HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) CAMBRIDGE BIOTECH HIV-1 WESTERN BLOT KIT For Detection of Antibodies to HIV-1

## NAME AND INTENDED USE

The Cambridge Biotech HIV-1 Western Blot Kit is an *in vitro* qualitative assay for the detection and identification of antibodies to Human Immunodeficiency Virus Type 1 (HIV-1). This more specific assay is used as a supplemental test with specimens (serum, plasma, or urine) that tested repeatedly reactive using a screening procedure (Enzyme Immunosorbent Assay). Urine specimens are to have been tested with the Calypte/Seradyn Sentinel HIV-1 Urine EIA Kit.

This professional use only kit contains a procedure for testing serum/plasma and a procedure for testing urine. A set of HIV-1 Urine Controls is required for testing urine specimens with the Cambridge Biotech HIV-1 Western Blot Kit. A specific Lot Number for the set of controls has been matched for use with this Western Blot Kit lot as identified on the outer package label.

**WARNING:**

- THE SENSITIVITY OF CAMBRIDGE BIOTECH HIV-1 WESTERN BLOT TESTING USING URINE SPECIMENS IS REDUCED COMPARED WITH TESTING USING SERUM OR PLASMA. FALSE NEGATIVES MAY OCCUR MORE OFTEN WHEN TESTING URINE SPECIMENS.
- THE SPECIFICITY OF CAMBRIDGE BIOTECH HIV-1 WESTERN BLOT TESTING USING URINE SPECIMENS IS REDUCED FOR HIGH RISK POPULATIONS AND PATIENTS WITH NON-HIV-1 MEDICAL CONDITIONS COMPARED WITH TESTING USING SERUM OR PLASMA. FALSE POSITIVES MAY OCCUR MORE OFTEN WHEN TESTING URINE SPECIMENS.
- WESTERN BLOT TEST RESULTS USING URINE SPECIMENS ARE NOT TO BE USED FOR SCREENING POTENTIAL BLOOD DONORS.
- ONLY THOSE URINE SPECIMENS REPEATEDLY REACTIVE ON THE CALYPTE/SERADYN SENTINEL HIV-1 URINE EIA KIT ARE TO BE FURTHER TESTED USING THE CAMBRIDGE BIOTECH HIV-1 WESTERN BLOT URINE PROCEDURE.

## SUMMARY AND EXPLANATION OF TEST

The Enzyme-Linked Immunosorbent Blot Technique ("Western Blot")[1,2] has been used to detect antibodies to Human Immunodeficiency Virus Type 1 (HIV-1), which has been recognized as the etiological agent of the Acquired Immunodeficiency Syndrome (AIDS).[2,3] The combination of electrophoretic separation of complex mixtures of antigens with the highly sensitive immunoblotting technique has been useful in characterizing the antigenic profile of HIV-1 and describing the immune response to this virus in exposed or infected persons.

The Cambridge Biotech HIV-1 Western Blot Kit, when used as directed in this insert, will detect antibodies to HIV-1 when present in human serum, plasma, or urine.[4,5] The position of bands on the nitrocellulose strips allows this antibody reactivity to be associated with specific viral antigens.

Persons demonstrating antibodies to HIV-1 should be referred for medical evaluation, which may include testing by other techniques. A clinical diagnosis of AIDS can be made only if a person meets the case definition of AIDS established by the Centers for Disease Control.[4]

Like most other chronic diseases, AIDS is a complicated multifactorial, multistep process, with HIV-1 infection being a principal component. Accurate diagnosis of HIV-1 infection is important in determining an individual's risk for developing AIDS. Accuracy is complicated by false-positive and false-negative (EIA) results. It would appear that in some limited infections, a compartmentalized response occurs in which expression of HIV-1 or its respective immune response is limited to a restricted number of organs and tissues.[17]

## CHEMICAL AND BIOLOGICAL PRINCIPLES OF THE PROCEDURE

The Cambridge Biotech HIV-1 Western Blot Kit is manufactured by Cambridge Biotech Corporation from HIV-1 propagated in an H9/HTLV-$III_B$ T-Lymphocyte cell line.[3] The partially purified virus is inactivated by treatment with psoralen and ultraviolet light, and detergent disruption. Specific HIV-1 proteins are fractionated according to molecular weight by electrophoresis on a polyacrylamide slab gel in the presence of sodium dodecylsulfate (SDS). The separated HIV-1 proteins are electrotransferred from the gel to a nitrocellulose membrane which is then washed, blocked (to minimize nonspecific immunoglobulin binding), and packaged. Individual nitrocellulose strips are incubated with specimens and controls. During incubation, if HIV-1 antibodies are present in the specimen, they will bind to the viral antigens present on the nitrocellulose strips. The strips are washed again to remove unbound material. Visualization of the human immunoglobulins specifically bound to HIV-1 proteins is accomplished *in situ* using a series of reactions with goat anti-human IgG conjugated with biotin, avidin conjugated with horseradish peroxidase (HRP), and the HRP substrate, 4-chloro-1-naphthol. If antibodies to any of the major HIV-1 antigens are present in the specimen in sufficient concentration, bands corresponding to the position of one or more of the following HIV-1 proteins (p) or glycoproteins (gp) will be seen on the nitrocellulose strip: p17, p24, p31, gp41, p51, p55, p66, gp120, gp160 (the number refers to the apparent molecular weight in kilodaltons).

## REAGENTS

Reagents for the Cambridge Biotech HIV-1 Western Blot Kit include:

1. NITROCELLULOSE STRIPS - Each NITROCELLULOSE STRIP contains separated, bound antigenic proteins from partially purified, inactivated HIV-1, in sufficient quantity to detect human antibodies. Bovine protein is present as a blocking agent. Strips are consecutively numbered (1 through 27).

2. NON-REACTIVE CONTROL - Inactivated normal human serum, non-reactive for antibodies to HIV-1 and non-reactive for hepatitis B surface antigen and antibodies to HCV. Contains 0.1% sodium azide and 0.005% thimerosal as preservatives.

3. WEAKLY REACTIVE CONTROL - Inactivated human serum containing a low titer of antibodies to HIV-1 antigens and non-reactive for hepatitis B surface antigen and antibodies to HCV. Contains 0.1% sodium azide and 0.005% thimerosal as preservatives.

4. STRONGLY REACTIVE CONTROL - Inactivated human serum containing a high titer of antibodies to HIV-1 antigens and non-reactive for hepatitis B surface antigen and antibodies to HCV. Contains 0.1% sodium azide and 0.005% thimerosal as preservatives.

5. WASH BUFFER - Supplied as a 20x concentrate. When diluted, this reagent contains 0.02 M tris, 0.1 M NaCl, 0.3% Tween 20, and 0.005% thimerosal as a preservative, at pH 7.4.

6. BLOTTING BUFFER - Supplied as a 10x concentrate. When diluted, this reagent contains 0.02 M tris, 0.1 M NaCl, heat-inactivated normal goat serum, and 0.01% thimerosal as a preservative, at pH 7.4.

7. CONJUGATE 1 - Biotinylated Goat Anti-human IgG (heavy and light chain) antibodies. Contains 0.002% thimerosal as a preservative.

8. CONJUGATE 2 - Avidin conjugated horseradish peroxidase. Contains 0.01% thimerosal as a preservative.

9. SUBSTRATE A - 7.8 mM solution of 4-chloro-1-naphthol in an alcohol solution.

10. SUBSTRATE B - Aqueous hydrogen peroxide solution (0.02%) in citrate buffer.

11. BLOTTING POWDER - nonfat dry milk (sterile).

12. HIV-1 Urine Controls - required for testing urine specimens with the Cambridge Biotech HIV-1 Western Blot Kit. These CONTROLS have been matched for use with this Western Blot kit lot, as identified on the outer package label.

Each set of urine controls contains:

- NEGATIVE URINE CONTROL
  Inactivated human urine negative for antibodies to HIV-1 antigens. A serum sample from the source was non-reactive for hepatitis B surface antigen and hepatitis C antibodies. (Supplied with Part No. 98076 only.) Contains 0.1% sodium azide as a preservative.

- LOW POSITIVE URINE CONTROL
  Inactivated human urine positive for antibodies to HIV-1 antigens. A serum sample from the source was non-reactive for hepatitis B surface antigen and hepatitis C antibodies. (Supplied with Part No. 98076 only.) Contains 0.1% sodium azide as a preservative.

- HIGH POSITIVE URINE CONTROL
  Inactivated human urine containing a high titer of antibodies to HIV-1 antigens. A serum sample from the source was non-reactive for hepatitis B surface antigen and hepatitis C antibodies. (Supplied with Part No. 98076 only.) Contains 0.1% sodium azide as a preservative.

## CAUTIONS

1. HANDLE ASSAY SPECIMENS, STRIPS, AND REACTIVE AND NON-REACTIVE CONTROLS AS IF CAPABLE OF TRANSMITTING AN INFECTIOUS AGENT. Inactivated HIV-1 antigen has been electrophoresed and transferred onto nitrocellulose. NON-REACTIVE, WEAKLY and STRONGLY REACTIVE CONTROLS, and the NEGATIVE, LOW and HIGH POSITIVE URINE CONTROLS have been inactivated by heat treatment. In addition, plasma used to produce the CONTROLS was shown to be non-reactive for hepatitis B surface antigen and for antibodies to HCV. A serum sample from the source of the urine used to manufacture the URINE CONTROLS was shown to be non-reactive for hepatitis B surface antigen and hepatitis C antibodies. However, no known test method can offer assurance that products derived from human source materials will not transmit infectious agents. **Therefore, these components must be handled as if they are capable of transmitting infectious agents.**

2. Do not pipet by mouth.

3. Wear disposable gloves throughout the procedure. Dispose of gloves as biohazard waste. Thoroughly wash hands after handling test reagents.

4. Wipe spills promptly with a 0.5% sodium hypochlorite solution (1/10 dilution of liquid household bleach). Contaminated materials should be disposed of as biohazard waste.

5. Dispose of all specimens and materials used in the Cambridge Biotech HIV-1 Western Blot Kit procedure as biohazard waste. The recommended method of disposal is autoclaving for a minimum of 1 hour at 121°C. Disposable materials may be incinerated. Mix liquid wastes with an equal volume of 5% sodium hypochlorite solution (liquid household bleach), allowing at least 60 minutes for disinfection.

6. Do not permit SUBSTRATE, especially 4-chloro-1-naphthol, to contact the skin. If contact occurs, flush with water.

7. The serum and urine CONTROLS contain sodium azide as a preservative. If these materials, either concentrated or diluted, are disposed of through a sink or other common plumbing systems, flush with generous amounts of water to prevent accumulation of potentially explosive compounds.

   In addition, consult the manual guideline, "Safety Management No. CDC-22, 'Decontamination of Laboratory Sink Drains to Remove Azide Salts'" (Centers for Disease Control, Atlanta, GA, April 30, 1976).

8. Avoid use of metal instruments in contact with SUBSTRATE B and WORKING SUBSTRATE SOLUTION, since metals can cause reduction in $H_2O_2$.

## PRECAUTIONS

**WARNING:**

- **The Cambridge Biotech HIV-1 Western Blot kit has reduced sensitivity and reduced specificity using urine specimens, compared with using serum or plasma specimens.**
- **FDA has licensed this test kit for use with serum, plasma, or urine. Use of this licensed test kit with specimens other than those specifically approved for use with this test kit may result in inaccurate test results.**

1. DO NOT INTERCHANGE REAGENTS (e.g. urine controls) BETWEEN KIT LOTS.

2. Reagents may have expiration dating beyond the kit expiration dating. DO NOT USE REAGENTS BEYOND KIT EXPIRATION DATING. Kit expiration dating is printed on the outer box label.

3. Do not use kit beyond its expiration date.

4. Avoid contamination of reagents when opening and withdrawing aliquots from primary vials. Keep all reagents refrigerated (2-8°C) when not in use.

5. Do not interchange vial or bottle caps and stoppers, this will lead to cross-contamination of reagents. Designate specific reservoirs for specific reagents.

6. Grossly contaminated specimens or strips may result in the development of dark spots on the strip which should not be interpreted. Careful attention must be given to the storage of specimens and kits to prevent this problem.

7. Shield WORKING SUBSTRATE SOLUTION from sunlight during preparation and use within 30 minutes of mixing.

8. Use reagent grade water (deionized water which is bacteria-free) to dilute reagents in order to avoid substances which may interfere with the assay.

9. Do not remove NITROCELLULOSE STRIPS from the storage tube until immediately before use. To prevent moisture from condensing inside the strip tube, open only AFTER the strips have reached room temperature (approximately 30 minutes). Close the tube immediately after removing strips for use.

10. Allow all kit reagents and materials to reach room temperature before use (approximately 30 minutes).

11. Use only the CONTROLS supplied with the kit for the analysis of serum, plasma, or urine specimens.

12. DO NOT CUT STRIPS. Narrower strips can lead to misinterpretation because strips may flip over in the incubation tray, or artifacts in the reaction zones may be mistaken for possible bands or may prevent recognition of positive bands.

13. Measure all reagents. Use extreme care and calibrated pipets with good quality tips when preparing WORKING CONJUGATE SOLUTIONS.

14. Discard bleach solution in trap prior to substrate preparation.

## PREPARATION OF REAGENTS

**NOTE: Allow reagents to reach room temperature before use (approximately 30 minutes).**

1. DILUTED WASH BUFFER

   a. Dilute 1 volume of WASH BUFFER (20x) with 19 volumes of reagent grade water. Mix well.

   b. DILUTED WASH BUFFER may be stored at room temperature for 3 months.

2. WORKING BLOTTING BUFFER

   a. WORKING BLOTTING BUFFER should be **prepared and used within 5 days.**

b. Dilute 1 volume of BLOTTING BUFFER (10x) with 9 volumes of reagent grade water. Mix well.

c. Use 1.0 g of BLOTTING POWDER per 20 mL of the diluted BLOTTING BUFFER prepared in Step 2b above. Mix thoroughly to dissolve the powder. If the entire kit is to be used within five days, add 9.0 g of BLOTTING POWDER to 180 mL of the diluted BLOTTING BUFFER (Step 2b). Store at 2-8°C.

3. WORKING CONJUGATE 1 SOLUTION

a. Refer to the SUPPLEMENTAL INSTRUCTIONS sheet for the dilution appropriate for the CONJUGATE 1 lot supplied with the kit.

b. WORKING CONJUGATE 1 SOLUTION should be **prepared fresh prior to use.**

4. WORKING CONJUGATE 2 SOLUTION

a. Refer to the SUPPLEMENTAL INSTRUCTIONS sheet for the dilution appropriate for the CONJUGATE 2 lot supplied with the kit.

b. WORKING CONJUGATE 2 SOLUTION should be **prepared fresh prior to use.**

5. WORKING SUBSTRATE SOLUTION

a. WORKING SUBSTRATE SOLUTION should be **prepared fresh prior to use.**

b. Prepare WORKING SUBSTRATE SOLUTION by mixing equal volumes of SUBSTRATE A and SUBSTRATE B. Mix well.

**Reagents Required (in mL)* for Various Number of Strips**

| | NUMBER OF STRIPS | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 3 | 6 | 9 | 15 | 20 | 27 |
| DILUTED WASH BUFFER | 20.0 | 60.0 | 120.0 | 180.0 | 300.0 | 400.0 | 540.0 |
| BLOTTING POWDER | 0.3g | 0.9g | 1.8g | 2.7g | 4.5g | 6.0g | 8.1g |
| WORKING BLOTTING BUFFER | 6.0 | 18.0 | 36.0 | 54.0 | 90.0 | 120.0 | 162.0 |
| WORKING CONJUGATE 1** | 2.0 | 6.0 | 12.0 | 18.0 | 30.0 | 40.0 | 54.0 |
| WORKING CONJUGATE 2** | 2.0 | 6.0 | 12.0 | 18.0 | 30.0 | 40.0 | 54.0 |
| SUBSTRATE A | 1.0 | 3.0 | 6.0 | 9.0 | 15.0 | 20.0 | 27.0 |
| SUBSTRATE B | 1.0 | 3.0 | 6.0 | 9.0 | 15.0 | 20.0 | 27.0 |

* Minimum volumes. Prepare a slight excess of each solution to compensate for loss during pipetting.
** See SUPPLEMENTAL INSTRUCTIONS sheet for Dilution Calculation.

## STORAGE INSTRUCTIONS

1. Store Cambridge Biotech HIV-1 Western Blot Kits and/or individual reagents at 2-8°C.

2. Unused NITROCELLULOSE STRIPS should be kept dry and in the dark, in their storage tube, at 2-8°C.

## INDICATIONS OF INSTABILITY OR DETERIORATION OF REAGENTS

Changes in the physical appearance of the reagents supplied may indicate instability or deterioration of these materials. SUBSTRATE A should be colorless. **If SUBSTRATE A shows color, it has become oxidized and should not be used.**

## KNOWN INTERFERING SUBSTANCES

Sodium azide interferes with horseradish peroxidase activity. Household bleach, when present in the wells at any stage of the assay, inhibits the ability of the assay to detect specific antibodies to HIV-1 viral components. When present at the substrate addition stage, this inhibition is accompanied by a visible fading of band intensity following the initial band development. The introduction of bleach into the test by any route (*e.g.*, aerosol, liquid, as a residual contaminant on trays) should be avoided.

## PROCEDURE FOR ASSAY OF SERUM AND PLASMA

## MATERIALS PROVIDED FOR SERUM AND PLASMA

Each Cambridge Biotech HIV-1 Western Blot Kit contains:

| | |
|---|---|
| • NITROCELLULOSE STRIPS | 27 Strips |
| • NON-REACTIVE CONTROL | 1 vial (Green Cap)<br>(160 $\mu$L/vial) |
| • WEAKLY REACTIVE CONTROL | 1 vial (Lavender Cap)<br>(160 $\mu$L/vial) |
| • STRONGLY REACTIVE CONTROL | 1 vial (Red Cap)<br>(160 $\mu$L/vial) |
| • WASH BUFFER (20x) | 1 Bottle<br>(60 mL/bottle) |
| • BLOTTING BUFFER (10x) | 1 Bottle<br>(18 mL/bottle) |
| • CONJUGATE 1 | 1 vial (Blue Cap)<br>(160 $\mu$L/vial) |
| • CONJUGATE 2 | 1 vial (Black Cap)<br>(160 $\mu$L/vial) |
| • SUBSTRATE A | 1 Bottle<br>(30 mL/bottle) |
| • SUBSTRATE B | 1 Bottle<br>(30 mL/bottle) |
| • BLOTTING POWDER | 1 Package<br>(9.0 g minimum) |
| • INCUBATION TRAYS | 3 Trays and Tops |

### MATERIALS REQUIRED - NOT PROVIDED

- ROCKER OR ROTARY PLATFORM
- PIPETS

- PIPETTORS AND TIPS
- ASPIRATOR WITH DISINFECTANT TRAP
- TWEEZERS OR FORCEPS
- 25 TO 30 WELL INCUBATION TRAY
  (IN LIEU OF SMALL TRAYS PROVIDED)

## SERUM AND PLASMA SPECIMEN COLLECTION AND PREPARATION

1. The Cambridge Biotech HIV-1 Western Blot Kit may be used with human serum or plasma. Reliability of the test results with grossly lipemic, hemolyzed, or cloudy specimens is not known.

2. Specimens should be stored at 2-8°C for up to two weeks. For longer intervals they should be frozen (-18°C or lower) prior to testing.

3. Avoid multiple freeze-thaw cycles.

4. Mix specimens well. Centrifuge if necessary to remove particulate matter prior to testing.

5. If specimens are shipped, they should be shipped in accordance with requirements for transporting etiological agents.

## SERUM AND PLASMA ASSAY PROCEDURE

**CAUTION:** When handling the incubation tray supplied with the kits, take care not to splash or mix specimens. Remove the lid carefully to prevent moisture which may condense on the lid from falling into the tray. Do not handle samples or sample loaded pipet tips over uncovered incubation trays. Splashing or aerosols may lead to cross-contamination of sample wells.

1. Bring all reagents to room temperature prior to use (approximately 30 minutes).

2. Add 2.0 mL of DILUTED WASH BUFFER to each well to be used.

3. Using forceps, carefully remove a NITROCELLULOSE STRIP from the vial in sequential order beginning with the number 1 and place numbered side up into a well containing DILUTED WASH BUFFER.

4. Place the tray on a rocker or rotary platform for 5 to 10 minutes at room temperature, then remove the buffer by aspiration.

5. Add 2.0 mL of WORKING BLOTTING BUFFER to each well.

6. Add 20 $\mu$L of each undiluted specimen or control to a well containing its assigned strip in WORKING BLOTTING BUFFER. CAUTION: Use a different pipet tip for each sample.

7. Cover the tray and incubate on a rocker or rotary platform overnight (14-20 hours) at room temperature (20 to 28°C).

8. Carefully uncover the tray to avoid splashing or mixing of specimens. Remove condensation or droplets on the incubation tray lid by rinsing with DILUTED WASH BUFFER or wiping with absorbent towels.

9. Aspirate the mixture from the wells into a trap containing disinfectant. Rinse aspirator tip with DILUTED WASH BUFFER, or reagent grade water between samples to avoid cross-contamination.

10. To each strip, add 2.0 mL of DILUTED WASH BUFFER and rock by hand several times. Remove buffer by aspiration.

11. Add 2.0 mL of DILUTED WASH BUFFER to each strip for a minimum of 5 minutes. Aspirate the wash buffer between washes. Repeat a second time. Perform all wash steps at room temperature on a rocker or rotary platform.

12. Add 2.0 mL of WORKING CONJUGATE 1 SOLUTION (prepared as directed in SUPPLEMENTAL INSTRUCTIONS sheet) to each well. Incubate for 60 minutes at room temperature on the rocker or rotary platform.

13. Aspirate the WORKING CONJUGATE 1 SOLUTION from the wells. Wash each strip three times for 5 minutes as in Step 11, above.

14. Add 2.0 mL of WORKING CONJUGATE 2 SOLUTION (prepared as directed in SUPPLEMENTAL INSTRUCTIONS sheet) to each well. Incubate for 60 minutes at room temperature on the rocker or rotary platform.

15. Aspirate the WORKING CONJUGATE 2 SOLUTION from the wells. Wash each strip three times for 5 minutes as in Step 11, above.

16. Add 2.0 mL of WORKING SUBSTRATE SOLUTION to each well and incubate at room temperature on the rocker or rotary platform for 10 to 15 minutes. The WEAKLY REACTIVE CONTROL must exhibit p24 and gp160 bands.

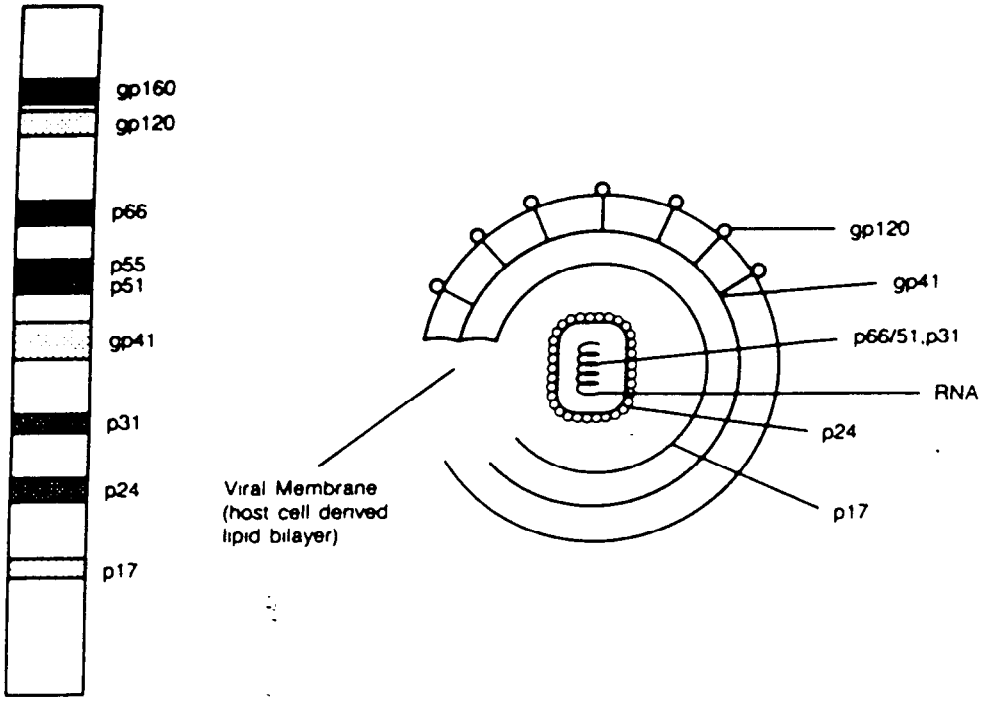
17. Aspirate,the WORKING SUBSTRATE SOLUTION and stop the reaction by rinsing the strips 2 or 3 times with at least 2mL of reagent grade water.

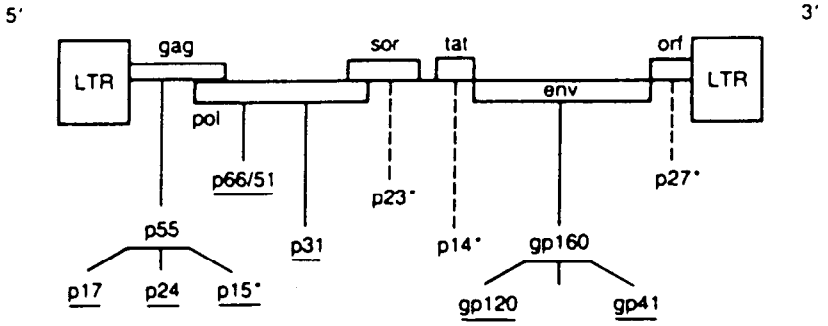
NOTE: Some specimens may cause spots to form on the strip due to precipitation. A cotton swab dipped in reagent grade water can be used to carefully remove the spots and allow for better visualization of results.

Air dry the strips between absorbent paper towels and score as described in the SERUM AND PLASMA INTERPRETATION OF RESULTS section. For best results and consistency, strips should be scored soon after air drying. The strips can be mounted and stored covered with cellophane. When mounting with tape, do not tape over developed bands. This will cause bands to fade.

18. If desired, the strips may be photographed using high resolution film. Developed strips will retain their color if stored in the dark. Exposure to light and air will eventually cause bands to fade.

## Figure 1
## Virus-Specific Bands for the Strongly Reactive Control




*These HIV-1 proteins generally do not appear with Cambridge Biotech HIV-1 Western Blot Kit

## SERUM AND PLASMA QUALITY CONTROL

The NON-REACTIVE and WEAKLY REACTIVE CONTROLS must be included with each run, regardless of the number of specimens tested or NITROCELLULOSE STRIPS used. The STRONGLY REACTIVE CONTROL is used to establish the location of bands and is to be included with the first run of specimens for each kit. The STRONGLY REACTIVE CONTROL need not be included in subsequent runs unless the strip is misplaced or faded.

In order for the results obtained from any run of specimens to be considered valid, the following conditions must be met:

1. NON-REACTIVE CONTROL: No bands should be visible on the NITROCELLULOSE STRIP used to test the NON-REACTIVE CONTROL.

2. STRONGLY REACTIVE CONTROL: All relevant molecular weight bands must be visible on the NITROCELLULOSE STRIP used to test the STRONGLY REACTIVE CONTROL. These bands are p17, p24, p31, gp41, p51, p55, p66, and gp160. A gp120 band may also be seen but is not a requirement for acceptable performance. Figure 1 provides a guide to the relative positioning of bands visualized with the Cambridge Biotech HIV-1 Western Blot Kit and permits identification of bands observed for the STRONGLY REACTIVE CONTROL.

3. WEAKLY REACTIVE CONTROL: The NITROCELLULOSE STRIP used to test the WEAKLY REACTIVE CONTROL provides a measure of the sensitivity of the Cambridge Biotech HIV-1 Western Blot Kit and must exhibit bands at p24 and gp160. Additional weak bands may appear but are not required to demonstrate acceptable performance. Figure 1 will permit identification of bands observed for the WEAKLY REACTIVE CONTROL.

## SERUM AND PLASMA INTERPRETATION OF RESULTS

The presence or absence of antibodies to HIV-1 in a specimen and the identity of any antibodies present are determined by comparison of each NITROCELLULOSE STRIP to the strips used for the NON-REACTIVE and WEAKLY REACTIVE CONTROLS tested with that run, and the strip used for the STRONGLY REACTIVE CONTROL tested once with the kit. The interpretation process requires three steps. First, each band which appears on the test strip must be identified based on the STRONGLY REACTIVE CONTROL strip. Second, each band is assigned a reactivity score based on its intensity. Third, the strip is interpreted based on the combination of band pattern and reactivity.

Figure 1 is used to locate and identify bands on the strip used with the STRONGLY REACTIVE CONTROL. This strip is then used to identify bands present on strips used to test specific specimens.

The major HIV-1 gene products that have been identified[5-10] are as follows (see Figure 1).

| | | |
|---|---|---|
| gp160 | - | Precursor of ENV glycoprotein |
| gp120 | - | Outer ENV glycoprotein |
| p66 | - | Reverse Transcriptase component of POL translate |
| p55 | - | Precursor of GAG proteins |
| p51 | - | Reverse Transcriptase component of POL translate |
| gp41 | - | Transmembrane ENV glycoproteins |
| p31 | - | Endonuclease component of POL translate |
| p24 | - | GAG protein |
| p17 | - | GAG protein |

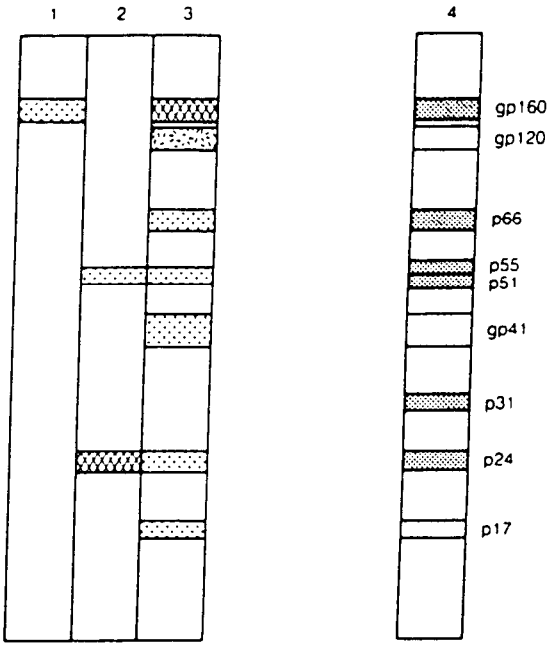
NOTE: The gp160 band may, in many cases, represent a multimer of gp41.[11] However, the presence of gp120 has been verified using specific mono and polyclonal antibodies. The primary response of most env reactive antibodies on Western Blot is to the transmembrane part whether it is a tetramer or derived from the precursor.

Intensity of bands present on strips used to test specific specimens may be scored as follows:

| **Intensity of Band** | **Reactivity Score** |
|---|---|
| • Absent | – |
| • Less than the intensity of p24 on the WEAKLY REACTIVE CONTROL strip. | ± |
| • At least as intense as p24 on the WEAKLY REACTIVE CONTROL strip but less intense than p24 on the STRONGLY REACTIVE CONTROL strip | + |
| • Greater than or equal to the intensity of p24 on the STRONGLY REACTIVE CONTROL strip. | ++ |

See examples given in Figure 2.

## Figure 2




**STRONGLY REACTIVE CONTROL**

| Strip | Interpretation | Bands Present |
|---|---|---|
| 1 | Indeterminate | gp160 |
| 2 | Indeterminate | p24, p55 |
| 3 | Positive | p17, p24, gp41, p55, p66, gp120, gp160 |
| 4 | STRONGLY REACTIVE CONTROL | p17, p24, p31, gp41, p51, p55, p66, gp120, gp160 |

Using the **STRONGLY REACTIVE CONTROL** as a reference for position and the p24 band on the **WEAKLY REACTIVE CONTROL** strip as a reference for intensity, each band on a strip should be assigned a reactivity score. When analyzing test specimens, it is helpful to place the control strips side-by-side with the unknown strips to facilitate the assignment of molecular weights and intensities of each band. The results of blotting are then interpreted as NEGATIVE, INDETERMINATE, or POSITIVE based on the pattern which is present, according to the following table:

| PATTERN | INTERPRETATION |
|---|---|
| No bands present. | NEGATIVE |
| Any bands present but pattern does not meet criteria for POSITIVE | INDETERMINATE |
| Any two or more of the following bands present: p24, gp41 and gp120/160. Each band has a reactivity score of + or greater. Commonly, the band at gp41 or gp160 is diffuse. Other viral bands may or may not be present. | POSITIVE |

The positive criteria follow the recommendations of the Centers for Disease Control (CDC)[12] and the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD).[13] These publications along with others[14-16] have suggested that the additional requirement for p31 reactivity is unnecessary.

Clinical studies with Cambridge Biotech HIV-1 Western Blot Kit have indicated that it is inappropriate to assign a POSITIVE interpretation to strips which display bands but lack any two of p24, gp41, or gp120/160 with a reactivity score of + or greater for each band present. It is known that persons who have recently seroconverted may display incomplete patterns but that they will develop increased reactivity (both number and intensity of bands) when followed for a period of four to six months. Most blots with POSITIVE results will have other virus-specific bands present including p17, p31, p51, p55, p66, and gp120.

Conversely, persons at low risk for infection may have nonspecific reactions on the blot particularly in regions corresponding to p17, p24, p55 and p66, which will persist but which do not evolve into more extensive patterns over time. Although nonspecific reactivity may sometimes be attributed to autoantibodies, it is possible that in some cases the pattern may represent a cross-reaction with another human retrovirus. Persons with HIV-1 infection may also present incomplete patterns due to the natural history of AIDS or other immunodeficiency states. In particular, it has been noted that AIDS patients lose antibody reactions to p24 and p31, and that infected infants may fail to seroconvert. In addition, infants may test POSITIVE for HIV-1 due to passive transfer of maternal antibodies which may persist for several months. Also, infected patients with malignancies and patients receiving immunosuppressive drugs may fail to develop a POSITIVE pattern.

Since reactivity of any degree with any of the virus-specific proteins (i.e., p24, p31, p66/51 or gp41/120/160) identified on the strip is presumptive evidence of antibodies to HIV-1, any such results (interpreted as INDETERMINATE) must be taken as suspicious and SHOULD TRIGGER REPEAT TESTING AND FOLLOW-UP TESTING. INDETERMINATE ASSAY RESULTS MUST NOT BE CONSIDERED POSITIVE OR NEGATIVE (See LIMITATIONS OF THE PROCEDURE section). The correct evaluation

in such situations must be based on subsequent blot testing and clinical evaluation. In such cases, INDETERMINATE blots may offer useful information.

Interpretation of two INDETERMINATE blots and one POSITIVE blot are shown in Figure 2.

In some instances, non-viral bands have been observed with certain specimens. These band patterns are usually not accompanied by any of the major viral bands of diagnostic significance (p24, gp41/120/160). The non-viral bands appear to be cell related with the most common in the molecular weight range of 70K, 51-55K (possible HLA DR) and 43K (possible HLA-ABC).

## LIMITATIONS OF THE SERUM AND PLASMA PROCEDURE

Optimal assay performance requires strict adherence to the assay procedure described in this insert. Deviations from the procedure may lead to aberrant results.

Highly reactive serum or plasma specimens may cause overdevelopment of the strip making interpretation of bands difficult due to strong background color development. Such specimens may be diluted 1:50 in WORKING BLOTTING BUFFER and the diluted specimen tested by the standard procedure described above.

Slight ambiguities exist in the designation of the molecular weights of the HIV-1 antigens. The designations listed in Figure 1 have been established by both internal testing with known markers and consensus of published literature.[5-10]

Although a blot POSITIVE for antibodies to HIV-1 indicates infection with the virus, a diagnosis of Acquired Immunodeficiency Syndrome or AIDS can only be made clinically if a person meets the case definition of AIDS established by the Centers for Disease Control.[4] POSITIVE blot results using any specimen type (serum, plasma, or urine) should be followed with additional testing. Such testing may rely on alternative test methods or specimen types. The clinical implications of antibodies to HIV-1 in an asymptomatic person are not known. However, a larger proportion of such persons have virus detectable in their peripheral blood and some will develop immunodeficiency. [9-10]

INDETERMINATE blots should not be used as the basis for diagnosis of HIV-1 infection. However, such findings may provide useful information in the context of medical evaluation for which clinical information is available.

DUE TO VARIATIONS IN TEST PERFORMANCE AND THE UNCERTAINTY ASSOCIATED WITH INDETERMINATE BLOTS, IT IS RECOMMENDED THAT ALL INDETERMINATE BLOTS BE REPEATED USING THE ORIGINAL SPECIMEN. DONORS WITH AN INDETERMINATE BLOT SHOULD BE RETESTED USING A FRESH SPECIMEN AFTER SIX MONTHS.

A NEGATIVE BLOT DOES NOT EXCLUDE THE POSSIBILITY OF INFECTION WITH HIV-1.

## SERUM AND PLASMA EXPECTED VALUES

The performance of the Cambridge Biotech HIV-1 Western Blot Kit was evaluated in clinical studies on high risk, low risk, and AIDS populations. Table A summarizes the prevalence of viral-specific bands in these groups.

### TABLE A
### FREQUENCY OF VIRUS SPECIFIC BANDS OF WESTERN BLOTS IN CLINICAL GROUPS

| Clinical Group | Number Studied | Number With Any Band | Percent Western Blots With Given Bands Present | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | p17 | p24 | p31 | gp41 | p51 | p55 | p66 | gp120/160 |
| Low Risk* | 364 | 168 | 54 | 70 | 70 | 75 | 64 | 71 | 75 | 79 |
| High Risk* | 149 | 56 | 71 | 86 | 95 | 96 | 91 | 84 | 98 | 96 |
| AIDS | 52 | 52 | 21 | 60 | 83 | 100 | 67 | 42 | 77 | 98 |

* Includes EIA negative and EIA repeatedly reactive specimens.

Table B summarizes the distribution of serum results when clinical groups are presented according to the procedure described in the SERUM AND PLASMA INTERPRETATION OF RESULTS section of this insert.

### TABLE B
### WESTERN BLOT INTERPRETATION AS DEFINED WITHIN CLINICAL GROUPS[a]

| Clinical Group | ELISA Status | Number Studied | Number (%) NEGATIVE | Number (%) INDETERMINATE | Number (%) POSITIVE |
|---|---|---|---|---|---|
| Low Risk | Negative[b] | 86 | 73(85) | 13(15) | 0(0) |
| | Positive[c] | 278 | 123(44) | 29(11) | 126(45) |
| High Risk | Negative[b] | 92 | 90(98) | 2(2) | 0(0) |
| | Positive[c] | 57 | 3(5) | 1(2) | 53(98) |
| AIDS | Unknown | 52 | 0(0) | 0(0) | 52(100) |

[a] Using criteria outlined under SERUM AND PLASMA INTERPRETATION OF RESULTS.
[b] Negative on up to five licensed ELISA tests.
[c] Repeatedly reactive on one or more of five licensed ELISA tests.

Additional testing of Cambridge Biotech HIV-1 Western Blot Kit was performed on specimens from persons with other clinical conditions that may cause reactions with proteins present. Specimens studied included those from persons with autoimmune disease, anti-HLA antibodies, polyclonal gammopathy, and viral infections other than HIV-1. Table C summarizes these results. There were no specimens in this group which would be interpreted as blot POSITIVE.

## TABLE C

## SUMMARY OF WESTERN BLOT RESULTS IN OTHER CLINICAL CONDITIONS

| | Number Studied | Number with Bands | p17 | p24 | p31 | gp41 | p51 | p55 | p66 | gp120/ 160 | Other[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Other Viral Diseases[b] | 124 | 11 | 0 | 9 | 0 | 0 | 0 | 2 | 0 | 0 | 1 |
| Infectious Diseases[c] | 5 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 |
| Elevated Bilirubin | 24 | 15 | 0 | 2 | 0 | 0 | 0 | 11 | 1 | 0 | 10 |
| Systemic Lupus Erythematosus | 19 | 6 | 0 | 1 | 0 | 0 | 0 | 5 | 0 | 0 | 4 |
| Hemolysis (in-vitro) | 22 | 6 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 0 |
| Rheumatoid Factor | 26 | 13 | 0 | 1 | 0 | 0 | 0 | 7 | 0 | 0 | 7 |
| Polyclonal Gammopathy | 17 | 8 | 0 | 1 | 0 | 1 | 1 | 6 | 2 | 2 | 5 |
| HLA Antibodies | 39 | 27 | 0 | 2 | 1 | 0 | 1 | 20 | 2 | 0 | 21 |

[a] Other bands were confined to the p45-50 or p70-80 regions.

[b] Other Viral Diseases include: 2 Epstein-Barr virus, 1 Varicella-zoster virus, 3 Cytomegalovirus, 3 Herpes Simplex, 1 Measles, 1 Mumps, 2 Coxsackie, 2 Adenovirus, 2 Influenza A and 107 samples from Africa, Jamaica, Trinidad, Hawaii and mainland U.S.A. which were strongly reactive for HTLV-I antibodies. Of those specimens reactive for HTLV-I, one had strong reactivity in the p24 region and some activity in the p55 region when tested with the Cambridge Biotech HIV-1 Western Blot Kit. An additional 7 of these specimens had a weak band at p24.

[c] Other Infectious Diseases include: 2 toxoplasmosis, 3 *M.* pneumoniae.

## SERUM AND PLASMA PRECISION

Precision of the Cambridge Biotech HIV-1 Western Blot Kit was evaluated in a field study at four cooperating centers in which the investigators tested a panel of sera in replicate on different days using different lots. At each site, each blot was independently interpreted by at least two readers. Refer to Table D.

**TABLE D**

**EXPECTED REACTIVITY SCORE AND PERCENT OF REPLICATE WESTERN BLOTS WITH REACTIVE BANDS EXPECTED REACTIVITY SCORE (% Reactive Replicates for Study Specimens)**

| | p17 | p24 | p31 | gp41 | p51 | p55 | p66 | gp120/ 160 |
|---|---|---|---|---|---|---|---|---|
| WEAKLY REACTIVE CONTROL | ± (16.1) | + (100.0) | - (0.0) | ± (71.0) | ± (61.3) | ± (93.5) | ± (98.4) | + (100.0) |
| Specimen 1 | ± (81.3) | ± (31.3) | + (100.0) | + (100.0) | + (100.0) | ± (50.0) | + (100.0) | + (100.0) |
| Specimen 2 | - (0.0) | + (100.0) | - (0.0) | + (100.0) | - (3.0) | + (100.0) | ± (29.7) | + (100.0) |
| Specimen 3 | + (100.0) | + (100.0) | + (100.0) | + (100.0) | - (0.0) | ± (90.7) | + (100.0) | + (100.0) |
| STRONGLY REACTIVE CONTROL | + (100.0) | + (100.0) | + (100.0) | + (100.0) | + (100.0) | + (100.0) | + (100.0) | + (100.0) |
| NEGATIVE CONTROL | - (0.0) | - (0.0) | - (0.0) | - (0.0) | - (0.0) | - (0.0) | - (0.0) | - (0.0) |

The results of this analysis were interpreted to show that bands more reactive than the WEAKLY REACTIVE CONTROL were highly reproducible, but bands weaker than the WEAKLY REACTIVE CONTROL were not highly reproducible.

# PROCEDURE FOR ASSAY OF URINE SPECIMENS

## MATERIALS PROVIDED FOR URINE SPECIMEN TESTING

**A set of HIV-1 Urine Controls is required for testing urine specimens with the Cambridge Biotech HIV-1 Western Blot Kit. A specific Lot Number for the set of controls has been matched for use with this Western Blot kit lot, as identified on the outer package label.**

| | |
|---|---|
| • NITROCELLULOSE STRIPS | 27 Strips |
| • WASH BUFFER (20x) | 1 Bottle<br>(60 mL/bottle) |
| • BLOTTING BUFFER (10x) | 1 Bottle<br>(18 mL/bottle) |
| • CONJUGATE 1 | 1 vial (Blue Cap)<br>(160 $\mu$L/vial) |
| • CONJUGATE 2 | 1 vial (Black Cap)<br>(160 $\mu$L/vial) |
| • SUBSTRATE A | 1 Bottle<br>(30 mL/bottle) |
| • SUBSTRATE B | 1 Bottle<br>(30 mL/bottle) |
| • BLOTTING POWDER | 1 Package<br>(9.0 g minimum) |
| • INCUBATION TRAYS | 3 Trays and Tops |
| • NEGATIVE URINE CONTROL<br>(Supplied with Part No. 98076 only.) | 1 Bottle (Translucent)<br>(10 mL/bottle) |
| • LOW POSITIVE URINE CONTROL<br>(Supplied with Part No. 98076 only.) | 1 Bottle (Amber)<br>(10 mL/bottle) |
| • HIGH POSITIVE URINE CONTROL<br>(Supplied with Part No. 98076 only.) | 1 Bottle (Amber with a blue bar)<br>(10 mL/bottle) |

**MATERIALS REQUIRED - NOT PROVIDED**

- ROCKER OR ROTARY PLATFORM
- PIPETS
- PIPETTORS AND TIPS
- ASPIRATOR WITH DISINFECTANT TRAP
- TWEEZERS OR FORCEPS
- 25 TO 30 WELL INCUBATION TRAY
  (IN LIEU OF SMALL TRAYS PROVIDED)

## URINE SPECIMEN COLLECTION AND PREPARATION

1. Polypropylene containers are recommended for collection, shipping or storing urine specimens.

2. Urine specimens without preservative or specimens preserved with Stabilur™ (R.P. Cargille Laboratories, Inc., Cedar Grove, NJ) may be used.

3. Urine specimens may be stored at 2-8°C for up to 12 months. DO NOT FREEZE. The reliability of results with frozen urine samples has not been demonstrated.

4. Mix specimens well prior to testing.

5. If specimens are shipped, they should be shipped in accordance with requirements for transporting etiological agents.

## URINE ASSAY PROCEDURE

CAUTION: When handling the incubation tray supplied with the kits, take care not to splash or mix specimens. Remove the lid carefully to prevent moisture which may condense on the lid from falling into the tray. Do not handle samples or sample loaded pipet tips over uncovered incubation trays. Splashing or aerosols may lead to cross-contamination of sample wells.

**WHENEVER URINE SPECIMENS ARE ASSAYED WITH THE CAMBRIDGE BIOTECH HIV-1 WESTERN BLOT, THE NEGATIVE, LOW POSITIVE, AND HIGH POSITIVE URINE CONTROLS MUST BE ASSAYED. SERUM CONTROLS MAY NOT BE USED TO EVALUATE URINE SPECIMENS.**

1. Bring all reagents to room temperature prior to use (approximately 30 minutes).

2. Add 2.0 mL of DILUTED WASH BUFFER to each well to be used.

3. Using forceps, carefully remove a NITROCELLULOSE STRIP from the vial in sequential order beginning with the number 1 and place numbered side up into a well containing DILUTED WASH BUFFER.

4. Place the tray on a rocker or rotary platform for 5 to 10 minutes at room temperature, then remove the buffer by aspiration.

5. Add 1.0 mL of WORKING BLOTTING BUFFER to each well which is to contain a urine control or urine specimen.

6. Add 1.0 mL of each undiluted urine control or urine specimen to a well containing its assigned strip in WORKING BLOTTING BUFFER. CAUTION: Use a different pipet tip for each sample.

7. Cover the tray and incubate on a rocker or rotary platform overnight (14-20 hours) at room temperature (20 to 28°C).

8. Carefully uncover the tray to avoid splashing or mixing of specimens. Remove condensation or droplets on the incubation tray lid by rinsing with DILUTED WASH BUFFER or wiping with absorbent towels.

9. Aspirate the mixture from the wells into a trap containing 5% household bleach as a disinfectant. Rinse aspirator tip with DILUTED WASH BUFFER, or reagent grade water between samples to avoid cross-contamination. Discard waste in the trap prior to substrate preparation.

10. Add 2.0 mL of DILUTED WASH BUFFER to each strip and incubate on a rocker or rotary platform for a minimum of 5 minutes. Aspirate the wash buffer. Repeat two additional times.

11. Add 2.0 mL of WORKING CONJUGATE 1 SOLUTION (prepared as directed in the SUPPLEMENTAL INSTRUCTIONS sheet) to each well. Incubate for 120 minutes at room temperature on the rocker or rotary platform.

12. Aspirate the WORKING CONJUGATE 1 SOLUTION from the wells. Wash each strip three times for a minimum of 5 minutes as in Step 10, above.

13. Add 2.0 mL of WORKING CONJUGATE 2 SOLUTION (prepared as directed in SUPPLEMENTAL INSTRUCTIONS sheet) to each well. Incubate for 120 minutes at room temperature on the rocker or rotary platform.

14. Aspirate the WORKING CONJUGATE 2 SOLUTION from the wells. Wash each strip three times for a minimum of 5 minutes as in Step 10, above.

15. Add 2.0 mL of WORKING SUBSTRATE SOLUTION to each well and incubate at room temperature on the rocker or rotary platform for 10-15 minutes. The LOW POSITIVE URINE CONTROL must exhibit a gp160 band.

16. Aspirate the WORKING SUBSTRATE SOLUTION and stop the reaction by rinsing the strips three (3) times with at least 2.0 mL of reagent grade water.

    NOTE: Some specimens may cause spots to form on the strip due to precipitation. A cotton swab dipped in reagent grade water can be used to carefully remove the spots and allow for better visualization of results.

    Air dry the strips between absorbent paper towels and score as described in the INTERPRETATION OF URINE RESULTS section. For best results and consistency, strips should be scored soon after air drying. The strips can then be mounted and stored covered with cellophane. When mounting with tape, do not tape over developed bands. This will cause bands to fade.

17. If desired, the strips may be photographed using high resolution film. Developed strips will retain their color if stored in the dark. Exposure to light and air will eventually cause bands to fade.

## URINE QUALITY CONTROL

The NEGATIVE, LOW POSITIVE and, HIGH POSITIVE URINE CONTROLS must be included with each run of urine specimens regardless of the number of specimens tested or NITROCELLULOSE STRIPS used. The HIGH POSITIVE URINE CONTROL is used to establish the location of the gp160 band for comparison with specimen strips.

In order for the results obtained from any run of urine specimens to be considered valid, the following conditions must be met:

1. NEGATIVE URINE CONTROL: No bands should be visible on the NITROCELLULOSE STRIP used to test the NEGATIVE URINE CONTROL.

2. HIGH POSITIVE URINE CONTROL: The NITROCELLULOSE STRIP used to test the HIGH POSITIVE URINE CONTROL must exhibit a band at gp160. Additional bands may appear but are not required to demonstrate acceptable performance. Figure 1 will permit location and identification of the gp160 band observed for the HIGH POSITIVE URINE CONTROL.

3. LOW POSITIVE URINE CONTROL: The NITROCELLULOSE STRIP used to test the LOW POSITIVE URINE CONTROL must exhibit a band at the gp160 region, and that band must be visibly less intense than the gp160 band on the HIGH POSITIVE URINE CONTROL. Other bands may be observed, but are not required to determine acceptable performance.

## INTERPRETATION OF URINE RESULTS

The presence or absence of antibodies to HIV-1 in a specimen and the identity of any antibodies present are determined by comparison of each NITROCELLULOSE STRIP to the strips used for the NEGATIVE, LOW POSITIVE, AND HIGH POSITIVE URINE CONTROL tested with that run.

The interpretation process requires three steps. First, each gp160 band which appears on a test strip must be identified based on the HIGH POSITIVE URINE CONTROL strip. Second, the gp160 band is assigned a reactivity score based on its intensity when compared to the gp160 band on the LOW POSITIVE URINE CONTROL strip. Third, the strip is interpreted based on the reactivity.

Figure 1 is used to locate and identify the gp160 band on the strip used with the HIGH POSITIVE URINE CONTROL. This strip is then used to identify the gp160 band location on strips used to test urine specimens.

Intensity of the gp160 band present on strips used to test specific specimens may be scored as follows:

| **Intensity Of Band** | **Reactivity Score** |
|---|---|
| • Absent | – |
| • Less than the intensity of the gp160 band on the LOW POSITIVE URINE CONTROL strip | ± |
| • Greater than or equal to the intensity of the gp160 band on the LOW POSITIVE URINE CONTROL strip. | + |

Interpret the result of blotting as NEGATIVE, INDETERMINATE, or POSITIVE based on the pattern which is present, according to the following table:

| **PATTERN** | **INTERPRETATION** |
|---|---|
| No bands present. | NEGATIVE |
| Any bands present but pattern does not meet the criterion for POSITIVE | INDETERMINATE |
| The presence of a gp160 band with a reactivity score of +. Other bands may or may not be present | POSITIVE |

Failure to obtain the established band criteria for the NEGATIVE, LOW POSITIVE, OR HIGH POSITIVE URINE CONTROLS may be indicative of unsatisfactory test performance. Such discrepancies may be related to the following sources of variability:

- Cross-contamination of negative control reagents with high titer specimens

- Cross-contamination of reactive control reagents with non-reactive specimens
- Improper equipment operation
- User reagent preparation

INDETERMINATE results must be taken as suspect and SHOULD TRIGGER REPEAT AND FOLLOW-UP TESTING. INDETERMINATE ASSAY RESULTS MUST NOT BE CONSIDERED POSITIVE OR NEGATIVE. The correct evaluation in such situations must be based on subsequent blot testing and clinical evaluation. In such cases, INDETERMINATE blots may offer useful information.

## LIMITATIONS OF THE URINE PROCEDURE

Optimal assay performance requires strict adherence to the assay procedure described in this insert. Deviations from the procedure may lead to aberrant results.

Slight ambiguities exist in the designation of the molecular weights of the HIV-1 antigens. The designations listed in Figure 1 have been established by both internal testing with known markers and consensus of published literature.[5-10]

Although a blot POSITIVE for antibodies to HIV-1 indicates infection with the virus, a diagnosis of Acquired Immunodeficiency Syndrome or AIDS can only be made clinically if a person meets the case definition of AIDS established by the Centers for Disease Control.[4] POSITIVE blot results using any specimen type (serum, plasma, or urine) should be followed with additional testing. Such testing may rely on alternative test methods or specimen types. The clinical implications of antibodies to HIV-1 in an asymptomatic person are not known. However, a larger proportion of such persons have virus detectable in their peripheral blood and some will develop immunodeficiency. [9-10]

INDETERMINATE blots should not be used as the basis for diagnosis of HIV-1 infection. However, such findings may provide useful information in the context of medical evaluation for which clinical information is available.

DUE TO VARIATIONS IN TEST PERFORMANCE AND THE UNCERTAINTY ASSOCIATED WITH INDETERMINATE BLOTS, IT IS RECOMMENDED THAT ALL INDETERMINATE BLOTS BE REPEATED USING THE ORIGINAL SPECIMEN. INDIVIDUALS WITH A BLOT THAT IS INDETERMINATE DUE TO A ± gp160 BAND SHOULD BE RETESTED AS SOON AS POSSIBLE USING A FRESH SPECIMEN. PERSONS WITH OTHER INDETERMINATE PATTERNS SHOULD BE RETESTED USING A FRESH SPECIMEN AFTER SIX MONTHS.

A NEGATIVE BLOT DOES NOT EXCLUDE THE POSSIBILITY OF INFECTION WITH HIV-1.

# PERFORMANCE CHARACTERISTICS

## PERFORMANCE STUDIES

Three studies were conducted to evaluate the performance of the Cambridge Biotech HIV-1 Western Blot Kit using a urine assay procedure. The performance was evaluated by comparing the results of urine specimens to the results of paired serum specimens tested with a licensed HIV-1 Western Blot Kit.

One study (Study 1) evaluated 696 archived urine specimens. The specimens were from low risk (N=200), high risk (N=37) and HIV-1 positive (N=377) populations. The HIV-1 positive populations included patients symptomatic (N=55) and asymptomatic (N=87) for HIV-1 infection, AIDS patients (N=115) and HIV-1 positive subjects from foreign sites (N=120) whose clinical status was unknown. Other specimens (N=82) were obtained and evaluated from subjects with medical conditions unrelated to HIV-1 infection that might result in antibodies cross-reactive with HIV-1 proteins.

The Cambridge Biotech HIV-1 Urine Western Blot results compared to serum Western Blot results are presented in Table E. Two additional studies (Study 2, Study 3) evaluated 1,240 prospectively collected urine specimens. Study 2 evaluated specimens from subjects whose HIV-1 clinical status was unclassified (N=197), subjects who were HIV-1 negative but at high risk of HIV-1 infection (N=51) and subjects with non-HIV related medical conditions (N=1). Study 3 evaluated low risk (N=315), high risk (N=303) and HIV-1 positive (N=175) populations, including AIDS patients. The HIV-1 positive populations included patients symptomatic (N=38) and asymptomatic (N=36) for HIV-1 infection and AIDS patients (N=101). Other specimens (N=198) were also obtained from subjects with unrelated medical conditions that might result in assay interference.

In the three studies combined, 1,936 paired urine and serum specimens collected from multiple geographical locations within the United States and from foreign sites were evaluated at four testing laboratories throughout the United States. The status of the subject was based upon the paired serum result or documented clinical status of the subject.

Two additional special studies were conducted using specimens from Study 3 to assess the performance of the Cambridge Biotech HIV-1 Western Blot kit. One evaluation involved Western blot testing of urine specimens paired to serum EIA non-reactive Western blot indeterminate specimens (N=109). The second evaluation involved urine Western blot testing of urine EIA repeatedly reactive specimens paired to serum EIA non-reactive specimens from uninfected individuals (N=114).

## SENSITIVITY STUDIES

### Sensitivity In HIV-1 Seropositive Individuals

The sensitivity of the Cambridge Biotech HIV-1 Western Blot Kit using urine was evaluated by comparing the urine results to the results obtained from testing paired serum specimens collected from individuals who were HIV-1 seropositive and from individuals clinically diagnosed as AIDS patients. The results of this study is shown in Table E.

In the combined studies, the Cambridge Biotech HIV-1 Western Blot Kit using urine obtained from the 215 patients clinically diagnosed with AIDS identified 213 of 215 (99.1%) patients as Western Blot positive. There were two (2) false negative urine Western Blot results in the AIDS population. The Cambridge Biotech HIV-1 Western Blot Kit using urine obtained from HIV-1 positive symptomatic, asymptomatic and unclassified groups correctly identified 533 of 533 (100%) patients as Western Blot positive. In the combined population of AIDS patients and other HIV-1 positive patients tested in this study, the Cambridge Biotech HIV-1 Western Blot Kit using urine correctly identified 746 of 748 (99.7%) patients as positive.

**Table E**

**Urine and Paired Serum Western Blot Results for Confirmed HIV-1 Seropositive Individuals and AIDS Patients (N=748)**

| Risk Group | N | Serum Western Blot Results[f] | | | Urine Western Blot Results | | |
|---|---|---|---|---|---|---|---|
| | | Pos[i] | Neg | Ind | Pos | Neg | Ind |
| AIDS[a] | 215 | 215 | 0 | 0 | 213 | 2 | 0 |
| Sympt[b] | 93 | 93 | 0 | 0 | 93 | 0 | 0 |
| Asympt[c] | 123 | 122 | 0 | 1[h] | 123 | 0 | 0 |
| Un-classified[d, e] | 317 | 296[g] | 0 | 1[h] | 317 | 0 | 0 |
| **Total** | 748 | 726 | 0 | 2 | 746 | 2 | 0 |

[a] One hundred and fifteen (115) specimens from Study 1, 100 specimens from Study 3.
[b] Fifty-five (55) specimens from Study 1, 38 from Study 3.
[c] Eighty-seven (87) specimens from Study 1, 36 from Study 3.
[d] One hundred twenty (120) specimens from Study 1, 197 from Study 2.
[e] The clinical status of these HIV-1 positive subjects was unknown.
[f] A licensed serum HIV-1 Western Blot Kit was used when testing serum specimens.
[g] Twenty (20) of the 316 specimens were from Uganda and were not confirmed by Western blot. The specimens were confirmed by a second manufacturer's EIA and by agglutination.
[h] The specimen did not meet the required band intensity criterion for a positive on serum Western blot and therefore was discordant with urine Western blot. However, the patient was known positive by previous clinical diagnosis.
[i] No intensity criterion associated with the LOW POSITIVE URINE CONTROL was used during this analysis.

## Sensitivity in High Risk Populations

The sensitivity was also determined in 391 individuals at high risk of HIV-1 infection but of unknown HIV status. Of the 391 high risk subjects tested, 327 were substance abusers and 64 were prostitutes, bisexuals, homosexuals, and other individuals with acknowledged risk factors. The results obtained are provided in Table F.

The results of testing urine specimens from these high risk populations showed that seventeen (17) of seventeen (17) (100%) urine Western blot specimens were correctly identified as positive when compared to the paired serum Western blot results. Of the twenty (20) urine Western blot positives, three (3) urine specimens were paired to serum EIA non-reactive specimens (urine false positives). While the significance of urine positivity in the absence of serum reactivity is not known, the results for these three samples must be classified as false positive in the absence of follow-up testing or clinical information to resolve the infection status of these individuals (see Table F, footnote f).

Sixty-nine (69) specimens were urine EIA repeatedly reactive and urine Western blot negative and were paired to serum EIA non-reactive specimens.

One (1) specimen was urine EIA repeatedly reactive and urine Western blot indeterminate and was paired to a serum EIA non-reactive specimen.

Three hundred (300) urine specimens were EIA non-reactive and Western blot negative and were paired to serum EIA non-reactive specimens. The one urine EIA non-reactive specimen that was Western blot indeterminate was paired to a serum EIA non-reactive specimen.

In this study the sensitivity of the urine Western blot was 100% (17 of 17) for seropositive individuals. In these high risk populations, the specificity of the urine Wesern blot for EIA repeatedly reactive urine specimens was 94.5% (69 of 73) for seronegative individuals.

Table F

## Comparison of Cambridge Biotech HIV-1 Western Blot Results Using Urine and Paired Serum for High Risk Populations (N=391)

| Risk Group | Serum EIA | | Serum Western Blot[b,d] | | Urine EIA | | Urine Western Blot | |
|---|---|---|---|---|---|---|---|---|
| | | N | | N | | N | | N |
| High | RR | 17 | Pos | 17 | RR | 90 | Pos[e] | 20[f] |
| | | | Ind | 0 | | | Ind | 1[a] |
| | | | Neg | 0 | | | Neg | 69 |
| | | | | | | | | |
| | NR | 374 | Pos | 0 | NR | 301 | Pos | 0 |
| | | | Ind | 123 | | | Ind | 1[c] |
| | | | Neg | 230 | | | Neg | 300[g] |
| | | | NT | 20[h] | | | NT | 0 |
| | | | UNR | 1[i] | | | UNR | 0 |

[a] Non viral bands
[b] A licensed HIV-1 Western Blot Kit was used when testing serum specimens.
[c] p24 only
[d] Serum EIA non-reactive specimens from Study 1 (N=20 drug abusers) were not tested by Western blot.
[e] No intensity criterion associated with the LOW POSITIVE URINE CONTROL was used during this analysis.
[f] Three (3) urine specimens were Western blot positive and paired to serum EIA non-reactive specimens (urine false positives).
[g] Two hundred twenty-nine (229) were correctly classified as negative when compared to the paired serum Western blot results.
[h] NT - Not Tested
[i] UNR - Unreadable

## Frequency of Virus Specific Bands in High Risk Populations

The frequency of virus specific bands and interpretation of results for urine specimens tested with the Cambridge Biotech HIV-1 Western Blot Kit from these high risk populations are presented in Table G.

The results show that in these combined high risk populations of 391 specimens, only one of the 301 (0.3%) urine EIA non-reactive specimens demonstrated any viral bands. The band present was p24, resulting in an indeterminate interpretation. Twenty (20) of 90 (22.2%) urine EIA repeatedly reactive specimens were identified as positive on the basis of the presence of the gp160 band. Seventeen (17) of the 20 (85%) urine Western blot positive specimens were paired to serum Western Blot confirmed HIV-1 positive specimens. One (1) urine specimen was paired to a serum Western Blot negative specimen and two (2) were paired to serum Western Blot indeterminate specimens. One (1) of the urine EIA repeatedly reactive specimens was indeterminate due to the presence of non-viral bands on the blot. Sixty-nine (69) urine EIA repeatedly reactive specimens were correctly classified as Western blot negative based on the paired serum results. The serum EIA results were non-reactive.

### Table G

### Frequency of Virus Specific Bands and Interpretation of Results of Urine Specimens from a High Risk Population Tested by the Cambridge Biotech HIV-1 Western Blot Kit (N=391[a])

| Urine EIA | Urine WB | | Frequency of Virus Specific Bands[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | N | p17 | p24 | p31 | gp41 | p51 | p55 | p66 | gp120 | gp160 |
| NR[c] | Pos[g] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| N=301 | Ind | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RR[d] | Pos[g] | 20 | 1 | 8 | 8 | 16 | 8 | 1 | 16 | 17 | 20[f] |
| N=90 | Ind | 1[e] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Thirty seven (37) specimens from Study 1, 51 specimens from Study 2, 303 specimens from Study 3.
[b] Band patterns for negative specimens do not appear in this table. By definition, negative specimens show no reactivity.
[c] NR indicates non-reactive in the Calypte Biomedical HIV-1 Urine EIA.
[d] RR indicates repeatedly reactive in the Calypte Biomedical HIV-1 Urine EIA.
[e] Specimen with non-viral bands.
[f] Three (3) of the 20 urine specimens had only a gp160 or gp120 and gp160 band present. All 3 were from Study 1.
[g] No intensity criterion associated with the LOW POSITIVE URINE CONTROL was used during this analysis.

## Frequency of Virus Specific Bands in AIDS Patients

Specimens from 215 AIDS patients were tested. Table H presents the frequency of viral specific bands observed and interpretation of results for these AIDS patients.

The results show that 213 of 215 (99.1%) specimens from AIDS patients were positive on the basis of the presence of a gp160 band when tested with the Cambridge Biotech HIV-1 Western Blot Kit.

**Table H**

**Frequency of Virus Specific Bands and Interpretation of Results of Urine Specimens from AIDS Patients (N=215[a])**

| Urine EIA | Urine WB | | Frequency of Virus Specific Bands[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | N | p17 | p24 | p31 | gp41 | p51 | p55 | p66 | gp120 | gp160 |
| RR[c] | Pos[e] | 213 | 50 | 104 | 130 | 199 | 114 | 44 | 166 | 210 | 213[d] |
| N=215 | Neg | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Ind | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] One hundred and fifteen (115) specimens from Study 1, 100 specimens from Study 3.
[b] Band patterns for negative specimens do not appear in this table. By definition, negative specimens show no reactivity.
[c] RR indicates repeatedly reactive in the Calypte Biomedical HIV-1 Urine EIA.
[d] Fifteen (15) of the 213 urine specimens had only a gp160 or gp120 and gp160 band present (11 from Study 1, 4 from Study 3).
[e] No intensity criterion associated with the LOW POSITIVE URINE CONTROL was used during this analysis.

## Frequency of Virus Specific Bands in Non-AIDS HIV-1 Positive Populations

The frequency of virus specific bands in non-AIDS HIV-1 populations (N=533) was also determined. The frequency of virus specific bands in HIV-1 symptomatic, asymptomatic and unclassified HIV-1 positive patients (120 foreign specimens from Study 1, 197 HIV-1 positive patients from Study 2) is given in Table I.

All 533 specimens were serum EIA repeatedly reactive and 531 serum Western blot positive, 2 were serum Western blot indeterminate. The results in Table I demonstrate the presence of a gp160 band in 533 of 533 of the HIV-1 positive urine specimens tested with the Cambridge Biotech HIV-1 Western Blot Kit, classifying all of the urine specimens as Western blot positive. There were no urine specimens that were negative or indeterminate in this population.

Table I

**Frequency of Virus Specific Bands and Interpretation of Results of Urine Specimens from Non-AIDS HIV-1 Positive Populations (N=533[a])**

| Urine EIA | Urine WB | | Frequency of Virus Specific Bands[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | N | p17 | p24 | p31 | gp41 | p51 | p55 | p66 | gp120 | gp160 |
| RR[d] | Pos[f] | 533 | 136 | 301 | 319 | 462 | 317 | 118 | 433 | 512 | 533[e] |
| N=533 | Ind | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Two hundred and sixty two (262) specimens from Study 1, 197 from Study 2, 74 from Study 3.

[b] Band patterns for negative specimens do not appear in this table. By definition, negative specimens show no reactivity.

[d] RR indicates repeatedly reactive in the Calypte Biomedical HIV-1 Urine EIA.

[e] Fifty (50) of the 533 urine specimens had only a gp160 or gp120 and gp160 band (37 from Study 1, 8 from Study 2, 5 from Study 3).

[f] No intensity criterion associated with the LOW POSITIVE URINE CONTROL was used during this analysis.

**Performance Using a Low Intensity Criterion**

The performance of Western blot testing when using an intensity criterion for the gp160 band was evaluated by testing a subset of urine samples from known HIV-1 positive patients (Table J). One hundred and seventy-two (172) urine samples were retested. The results were interpreted by comparing the intensity of the gp160 band of the specimen with that of the LOW POSITIVE URINE CONTROL. The testing was performed, and results were interpreted at two different sites. A description of the known HIV-1 positive urine specimens that were retested and the results obtained at both sites are presented in Tables J and K.

The results show that the 2 sites read the same specimen as indeterminate. The sites differed on their interpretation for a second specimen (Site 1 read the sample as positive, Site 2 read the specimen as indeterminate). Therefore, the frequency of indeterminate results in a sample population of 172 HIV-1 EIA RR urine specimens ranged from 0.6% (1 of 172 specimens read at Site 1) to 1.2% (2 of 172 specimens read at Site 2).

The sensitivity of the Western blot using the intensity criterion associated with the LOW POSITIVE URINE CONTROL ranged from 98.3% (169/172) to 98.8% (170/172).

**Table J**
**Description of HIV-1 Positive Specimens Retested On The Cambridge Biotech HIV-1 Western Blot Kit**

| Study (N) | Study Group | Clinical Condition ((Samples Collected (N)) | N Tested |
|---|---|---|---|
| 1 (100) | HIV-1 Seropositive Women | AIDS (26)<br>HIV-1 Symptomatic (38)<br>HIV-1 Asymptomatic (36) | 25[a]<br>37[b]<br>35[c] |
| 2 (75) | AIDS Patients | AIDS (75) | 75 |
| Total (175) | | 175 | 172 |

[a] One of the 26 AIDS patient specimens had insufficient volume to test by Western blot.
[b] One of the 38 HIV-1 positive symptomatic patient specimens had insufficient volume to test by Western blot.
[c] One of the 36 HIV-1 positive asymptomatic patient specimens had insufficient volume to test by Western blot.

**Table K**
**Western Blot Results for Known HIV-1 Positive Patients Using a gp160 Band Intensity Criterion**

| | No Intensity Criterion | | | Using gp160 Band Intensity Criterion Western Blot Results | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| N | Prior[a] Western Blot Result | | | Site 1 | | | Site 2[g] | | |
| Tested | Pos | Ind | Neg | Pos | Ind | Neg | Pos | Ind | Neg |
| 172[b] | 171 | N/A | 1[c] | 170 | 1[d] | 1[e] | 169 | 2[f] | 1[e] |

[a] "Prior" indicates data from Table E: analysis without an intensity criterion.
[b] Three of the original 175 urine specimens had insufficient volume to test by Western blot.
[c] Specimen was borderline EIA repeatedly reactive (S/CO range 0.972 to 1.916), negative on Western blot in Table E.
[d] Specimen is +/- gp160/+gp120 only. The EIA testing associated with Western blot testing using Intensity Criterion was 1.284 S/CO. The specimen was previously urine Western blot false negative (Table E).
[e] Specimen was urine EIA non-reactive and false negative (S/CO=0.825).
[f] One Indeterminate was the specimen identified in footnote d.
[g] The blots were interpreted approximately 7 days after processing.

## SPECIFICITY STUDIES

### Specificity in Low Risk Groups

The specificity of the Cambridge Biotech HIV-1 Western Blot Kit was assessed by testing specimens from 515 EIA seronegative subjects at low risk for HIV-1 infection. The subjects were insurance applicants. Insurance applicants are presumed to be at low risk for HIV-1 infection. The results obtained from testing paired urine and serum specimens from low risk uninfected individuals by Western blot are provided in Table L.

The results show that in this low risk population, the specificity of the Cambridge Biotech HIV-1 Urine Western Blot was 100% (515 of 515 urine specimens were Western blot negative). There were no (0%) urine Western blot indeterminate or positive specimens.

**Table L**

**Comparison of Cambridge Biotech HIV-1 Western Blot Results Using Urine and Paired Serum Specimens from Low Risk Populations (N=515)**

| | | Serum | | | Urine | | | |
|---|---|---|---|---|---|---|---|---|
| Risk Group | N | Western Blot Results[c] | | | EIA | Western Blot Results | | |
| | | Pos | Neg | Ind | RR | Pos[d] | Neg | Ind |
| Low[a] | 200[b] | NT | NT | NT | 0 | 0 | 200 | 0 |
| | 315 | 0 | 284 | 31 | 1 | 0 | 315 | 0 |

[a] Two hundred (200) specimens from Study 1, 315 specimens from Study 3.
[b] The 200 archived serum specimens (Study 1) were EIA NR and were not tested by Western blot.
[c] A licensed HIV-1 Western Blot Kit was used when testing serum specimens.
[d] No intensity criterion associated with the LOW POSITIVE URINE CONTROL was used during this analysis.

### Frequency of Virus Specific Bands in Low Risk Groups

The frequency of virus specific bands and interpretation of results for urine specimens tested with the Cambridge Biotech HIV-1 Western Blot Kit for these low risk groups are presented in Table M.

The results show that in these populations of 515 low risk subjects, none of the urine specimens were positive or indeterminate when tested with the Cambridge Biotech HIV-1 Western Blot Kit. All 515 serum specimens were EIA non reactive; 200 serum specimens were not tested by Western blot; of 315 serum specimens that were tested by Western blot, 284 were negative, 31 were indeterminate, and none were positive. All 515 urine specimens were identified as Western blot negative, including the one urine specimen that was EIA initially reactive. This demonstrates the high specificity of the Cambridge Biotech HIV-1 Western Blot Kit for urine in low risk populations.

## Table M

### Frequency of Virus Specific Bands and Interpretation of Results of Urine Specimens from Low Risk Populations Tested by the Cambridge Biotech HIV-1 Western Blot Kit (N=515[a])

| Urine EIA | Urine WB | | Frequency of Virus Specific Bands[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | N | p17 | p24 | p31 | gp41 | p51 | p55 | p66 | gp120 | gp160 |
| NR[c] | Pos | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| N=514 | Ind | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RR[d] | Pos | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| N=1[e] | Ind | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Two hundred (200) specimens from Study 1, 315 specimens from Study 3.
[b] Band patterns for negative specimens do not appear in this table. By definition, negative specimens show no reactivity.
[c] NR indicates non-reactive in the Calypte Biomedical HIV-1 Urine EIA.
[d] RR indicates repeatedly reactive in the Calypte Biomedical HIV-1 Urine EIA.
[e] The one urine specimen initially reactive in the EIA was not repeat tested.

### Frequency of Virus Specific Bands in Other Groups

Two additional special studies of paired urine and serum specimens were collected for evaluation by Western blot. The first evaluation involved Western blot testing of urine specimens paired to serum EIA non-reactive Western blot indeterminate specimens (N=109). The purpose of the study was to demonstrate the specificity of the urine Western blot for those samples that are repeatedly reactive on the urine EIA. The results are shown in Table N.

In this evaluation, 4 of 109 (3.7%) urine specimens paired to serum Western blot indeterminate specimens were urine Western blot indeterminate. None were positive. One hundred and five (105) of 109 (96.3%) were negative. These results show the specificity of the Cambridge Biotech HIV-1 Western Blot Kit for samples from uninfected individuals who are repeatedly reactive (false positive) on the urine EIA.

## Table N

### Frequency of Virus Specific Bands and Interpretation of Results of Urine Specimens Paired to Serum Western Blot Indeterminate Specimens from Uninfected Individuals Tested by the Cambridge Biotech HIV-1 Western Blot Kit (N=109[a])

| Urine EIA | Urine WB | | Frequency of Virus Specific Bands | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | N[d] | p17 | p24 | p31 | gp41 | p51 | p55 | p66 | gp120 | gp160 |
| NR[b] | Pos | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| N=66 | Ind | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| RR[c] | Pos[e] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| N=43 | Ind | 3 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |

[a] All 109 specimens were from Study 3.
[b] NR indicated non-reactive in the Calypte Biomedical HIV-1 Urine EIA.
[c] RR indicates repeatedly reactive in the Calypte Biomedical HIV-1 Urine EIA.
[d] Includes specimens with non-viral bands.
[e] No intensity criterion associated with the LOW POSITIVE URINE CONTROL was used during this analysis.

The second evaluation involved urine Western blot testing of urine EIA repeatedly reactive specimens paired to serum EIA non-reactive specimens from uninfected individuals (N=114). The purpose of this study was to assess the utility of the urine Western blot for individuals who are not infected but whose urine specimens are repeatedly reactive using the HIV-1 EIA. The frequency of virus specific bands in each group of urine specimens tested is demonstrated in the Table O.

Of the 114 urine EIA repeatedly reactive specimens paired to serum EIA non-reactive specimens, 109 of 114 (95.6%) were Western blot negative, 5 of 114 (4.4%) were Western blot indeterminate and none were positive. This demonstrates the ability of the Cambridge Biotech HIV-1 Western Blot Kit to resolve urine EIA repeatedly reactive specimens from uninfected individuals as negative or indeterminate.

Table O

**Frequency of Virus Specific Bands and Interpretation of Urine EIA False Positive Urine Specimens Tested by the Cambridge Biotech HIV-1 Western Blot Kit (N=114[a])**

| Urine EIA | Urine WB | | Frequency of Virus Specific Bands | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | N | p17 | p24 | p31 | gp41 | p51 | p55 | p66 | gp120 | gp160 |
| RR[b] | Pos | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| N=114 | Ind | 5 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] All 114 specimens were from Study 3.
[B] RR indicates repeatedly reactive in the Calypte Biomedical HIV-1 Urine EIA.

**Specificity in Subjects with Medical and Other Conditions**

The specificity of the Cambridge Biotech HIV-1 Western Blot Kit was evaluated using urine from individuals with medical conditions unrelated to HIV-1 and from individuals with potential interfering substances in their urine. The results of testing urine specimens from these individuals with the Cambridge Biotech HIV-1 Urine Western Blot Kit are provided in Table P.

The results obtained from testing urine specimens collected from patients with diseases and potentially interfering substances showed that 2 urine specimens were urine EIA repeatedly reactive and Western blot positive and were paired to serum EIA repeatedly reactive and Western blot positive specimens.

For subjects with non-HIV-1 medical conditions, the specificity of the urine Western blot for EIA repeatedly reactive urine specimens in this study was 90.1% (100/111).

Table P

**Comparison of Cambridge Biotech HIV-1 Western Blot Results Using Urine and Paired Serum in Populations with Disease and Potentially Interfering Substances (N=281)**

| Group | N | Urine EIA Results | | Urine Western Blot Results | | |
|---|---|---|---|---|---|---|
| | | RR | NR | Pos[j] | Neg | Ind |
| Autoimmune[a] | 25 | 7 | 18 | 0 | 25 | 0 |
| Kidney/Liver[b] | 59 | 32 | 27 | 0 | 55 | 4 |
| STD[c] | 37 | 5 | 32 | 0 | 37 | 0 |
| Urine Cond.[d] | 47 | 22 | 25 | 1 | 45 | 1 |
| Pregnant[e] | 63 | 25 | 38 | 1 | 59 | 3 |
| Neoplasms[f] | 35 | 17 | 18 | 0 | 35 | 0 |
| Multiple Transfusions[g] | 13 | 5 | 8 | 0 | 10 | 3 |
| Multiparous[h] | 2 | 0 | 2 | 0 | 2 | 0 |
| **Total** | 281 | 113 | 168 | 2[m] | 268 | 11[k] |

[a] Twenty (20) specimens from Study 1, 5 specimens from Study 3.
[B] Twenty (20) specimens from Study 1, 39 specimens from Study 3.
[c] STD = Sexually transmitted disease. Twenty two (22) specimens from Study 1, 15 specimens from Study 3.
[d] Twenty (20) specimens from Study 1, 27 specimens from Study 3.
[e] Sixty three (63) specimens from Study 3.
[f] Thirty five (35) specimens from Study 3.
[g] One (1) specimen from Study 2, 12 specimens from Study 3.
[h] Two (2) specimens from Study 3.
[j] No intensity criterion associated with the LOW POSITIVE URINE CONTROL was used during this analysis.
[k] These specimens were all urine EIA repeatedly reactive.
[m] These two specimens were true serum positive as confirmed.

## ANALYTICAL SENSITIVITY

### Dilution of Paired Urine and Serum Specimens

Ten (10) paired urine and serum specimens from HIV-1 positive individuals were tested in a dilution study using the Cambridge Biotech HIV-1 Western Blot Kit. The urine specimens were tested at dilutions of 1:2, 1:10, 1:50, 1:500 and 1:1,000. The serum specimens were tested at dilutions of 1:101, 1:1,000, 1:5,000, 1:25,000 and 1:50,000. The urine specimens were tested according to the urine procedure. The serum specimens were tested according to the manufacturer's package insert instructions. The major viral bands (gp160, gp120, gp41 and p24) were observed for presence or absence on Western blot at each dilution. The results are reported as the last dilution at which a band is visible on Western blot without comparison to an intensity criterion.

A side by side comparison of the urine and serum dilutions are presented in Table Q. The comparison shows that the gp160 band can be observed at a higher dilution than the gp120, gp41 or p24 bands for both urine and serum specimens tested in dilution series.

The difference in the analytical sensitivity between the licensed Serum Western Blot Kit and the Urine Western Blot Kit for antibodies to different proteins ranged from a ratio of greater than 50 to greater than 25,000.

## Table Q

**Comparison of Dilutions of HIV-1 Positive Paired Urine & Serum Specimens Tested on Cambridge Biotech Corporation's HIV-1 Western Blot Kit**

**Last Dilution At Which Major Viral Band is Present**

| | | | Major Viral Bands^ | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | gp160 | | gp120 | | gp41 | | p24 | |
| No. | Specimen No. | Clinical Classification* | Urine | Serum | Urine | Serum | Urine | Serum | Urine | Serum |
| 1 | SP00000604 | AIDS | 1:500 | >1:50,000 | 1:10 | >1:50,000 | 1:10 | >1:50,000 | - | - |
| 2 | SP00000606 | AIDS | 1:50 | >1:50,000 | 1:2 | >1:50,000 | 1:2 | >1:50,000 | - | 1:1,000 |
| 3 | SP00001709 | HIV-1 Symptomatic | 1:10 | >1:50,000 | - | >1:50,000 | - | >1:50,000 | - | 1:10,000 |
| 4 | SP00001752 | HIV-1 Symptomatic | 1:50 | >1:50,000 | 1:2 | >1:50,000 | 1:2 | >1:50,000 | 1:10 | >1:50,000 |
| 5 | SP00001783 | HIV-1 Asymptomatic | 1:50 | >1:50,000 | 1:2 | >1:50,000 | - | 1:10,000 | - | 1:101 |
| 6 | CL1 | F-TL | 1:50 | >1:50,000 | - | >1:50,000 | - | >1:50,000 | - | >1:50,000 |
| 7 | CL35 | F-TL | 1:10 | >1:50,000 | - | 1:25,000 | - | 1:10,000 | - | 1:10,000 |
| 8 | CL83 | F-TL | 1:1,000 | >1:50,000 | 1:50 | >1:50,000 | 1:10 | >1:50,000 | >1:1,000 | >1:50,000 |
| 9 | 175 | F-TZ | 1:500 | >1:50,000 | 1:50 | >1:50,000 | 1:10 | >1:50,000 | 1:1,000 | >1:50,000 |
| 10 | 3.030751 | F-Abidj | 1:10 | >1:50,000 | - | >1:50,000 | - | 1:25,000 | 1:10 | >1:50,000 |

* F-TL = Foreign specimen from Thailand

* F-TZ = Foreign specimen from Tanzania

• F-Abidj = Foreign specimen from Abidjan

^ (-) indicates these bands were not present for the original serum specimen diluted 1:101 according to the serum Western Blot procedure and for the original urine specimen diluted 1:2 according to the Western Blot procedure for urine.

## REPRODUCIBILITY

The reproducibility of the Cambridge Biotech HIV-1 Western Blot Kit was evaluated by testing a panel of urine specimens at three (3) geographically separate sites. A panel of 12 specimens of defined viral reactivity was provided to the three sites for evaluation.

The panel consisted of specimens strongly reactive and weakly reactive for antibodies to HIV-1 and specimens non-reactive for antibodies to HIV-1. Each panel member was tested in duplicate on three different lots of the Cambridge Biotech HIV-1 Western Blot Kit. The testing was performed by at least two different operators at each of 3 sites over multiple days.

In addition to the 12 specimens, a high positive urine control, low positive urine control and a negative urine control were provided for testing with each kit.

The results of this analysis demonstrate the reproducibility of the Cambridge Biotech HIV-1 Western blot for urine specimens with HIV-1 antibody activity to the gp160 viral gene product at the limit of visual detection. The combined results of testing are provided in Table R.

Table R

## Number of Replicates of Western Blots With Reactive Bands (% Reactive Replicates)

| Spec/ Res[a] | # of Reps[h] | p17 | p24 | p31 | gp41 | p51 | p55 | p66 | gp120 | gp160 |
|---|---|---|---|---|---|---|---|---|---|---|
| HPC[b] | 24 | 23 (96) | 24 (100) | 24 (100) | 24 (100) | 15 (63) | 13 (54) | 24 (100) | 24 (100) | 24 (100) |
| LPC[c] | 24 | 3 (13) | 24 (100) | 23 (96) | 17 (71) | 6 (25) | 0 (0) | 21 (88) | 24 (100) | 24 (100) |
| NC[d] | 24 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| 1 Neg[e] | 48 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| 2 Neg[e] | 48 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| 3 S. Pos[f] | 48 | 16 (33) | 2 (4) | 48 (100) | 20 (42) | 28 (58) | 18 (38) | 48 (100) | 48 (100) | 48 (100) |
| 4 S. Pos[f] | 40 | 40 (100) | 40 (100) | 40 (100) | 40 (100) | 34 (85) | 22 (55) | 40 (100) | 40 (100) | 40 (100) |
| 5 W. Pos[g] | 40 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 14 (35) | 0 (0) | 40 (100) | 40 (100) |
| 6 W. Pos[g] | 40 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 40 (100) | 40 (100) |
| 7 W. Pos[g] | 48 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 28 (58) | 48 (100) | 48 (100) |
| 8 W. Pos[g] | 48 | 0 (0) | 46 (96) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 48 (100) | 48 (100) |
| 9 W. Pos[g] | 48 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 2 (4) | 48 (100) | 48 (100) |
| 10 W. Pos[g] | 48 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 48 (100) | 48 (100) |
| 11 W. Pos[g] | 48 | 0 (0) | 24 (50) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 48 (100) | 48 (100) |
| 12 W. Pos[g] | 48 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 48 (100) | 48 (100) |

[a] Spec/Res=Specimen ID#/Expected Result
[b] HPC=High Positive Urine Control
[c] LPC=Low Positive Urine Control
[d] NC=Negative Urine Control
[e] Neg=Negative Specimen
[f] S Pos=Strong Positive Specimen
[g] W Pos=Weak Positive Specimen
[h] Reps=Replicates

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# ATTACHMENT 9

VIEW IN FRAME

**Important note:** Information in this article was accurate in 1987. The state of the art may have changed since the publication date.

U.S. Food and Drug Administration

# AIDS Test Kit Licensed

*Food and Drug Administration, U.S. Department of Health and Human Services - April 20, 1987*
*Susan Cruzan: (301) 443-3285*

The Food and Drug Administration today licensed a commercial test kit -- previously used only in a research setting -- for screening blood and for validating an initial screening of donated blood for antibodies to the virus that causes AIDS, acquired immunodeficiency syndrome.

**FDA** Commissioner Frank E. Young, M.D., Ph.D., said, "This validation kit will help re-instate some previously ineligible donors and thus will increase the supply of blood available to blood banks and patients needing transfusions. The kit should increase our confidence in the accuracy of our findings."

The initial test of donated blood uses a screening test called ELISA (enzyme-linked immunosorbent assay). To protect the public, the ELISA was designed for maximum sensitivity to eliminate virtually all questionable blood and plasma. However, this increased sensitivity results in some nonspecific or false positive readings.

A test called "the Western blot" can then be performed to validate the earlier finding of a positive ELISA. Previously, the Western blot test procedure had been used in a research setting to identify specific AIDS antibodies. The Western blot kit announced today standardizes the procedure for use by blood bank personnel.

The standardization and licensing of this additional, more specific test kit can help solve a blood bank problem resulting when donors who are AIDS antibody-negative have tested falsely positive by the ELISA test. Under current guidelines, when a donor tests positive to the ELISA, his or her blood is not used for transfusion. Many of these positive ELISA tests are thought to be "false positive," due to reactions to test components (other than viral proteins) or to other technical problems.

A plan to use validation tests to reenter donors into the donor pool was discussed Feb. 13 by the FDA Blood Products Advisory Committee.

The FDA proposed a plan that involves retesting with the ELISA and Western blot on two different samples of blood taken six months apart. If all tests on the freshly drawn samples are negative (do not show antibody presence) the donor would be reentered.

FDA will soon recommend to the blood community a specific procedure for donor reentry.

Robert E. Windom, M.D., HHS assistant secretary for health, emphasized that individuals with antibody-positive Western blot results should be referred for medical evaluation, which may include additional testing. The significance of antibodies in an asymptomatic individual is not known. However, many people with AIDS virus antibodies will develop AIDS with the passage of time.

The approved Biotech/DuPont HIV Western blot kit will be manufactured by Biotech Research Laboratories, Inc. of Rockville, Md., and distributed by E.I. Du Pont de Nemours and Company of Wilmington, Del.

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FD870401

SOURCE: Food and Drug Administration (**FDA**).

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# ATTACHMENT 10

# Laboratory and Epidemiologic Evaluation of an Enzyme Immunoassay for Antibodies to HTLV-III

John W. Ward, MD; Alfred J. Grindon, MD; Paul M. Feorino, PhD; Charles Schable, MS; Michael Parvin, MT; James R. Allen, MD, MPH

**The enzyme immunoassays (EIAs) for antibody to human T-cell lymphotropic virus type III (HTLV-III) were rapidly adopted for screening donated blood and plasma. To evaluate the significance of a positive EIA reaction, test performance was examined in a blood bank screening program. Specimens were tested by EIA, Western blot assay, and HTLV-III/lymphadenopathy-associated virus (LAV) culture. The EIA was positive in 0.25% of 67 190 blood donations. Specimens were categorized and 57.3% had low (weak) reactivity, 12.7% had moderate reactivity, and 30.0% had high reactivity. Highly reactive specimens were strongly associated with a positive Western blot or culture (86.7%) in contrast to moderately and weakly reactive specimens (1.9%). Twenty-five of 29 donors interviewed with a highly reactive EIA had risk factors for HTLV-III/LAV infection. Risk factors were not identified for 74 of 75 interviewed donors with specimens of lower reactivity. The minimum calculated specificity was 99.82%. The use of the HTLV-III EIA has virtually eliminated the use of blood and plasma from HTLV-III/LAV infected donors.**

(*JAMA* 1986;256:357-361)

BLOOD transfusions have been implicated as the source of infection for 1.7% of all cases of acquired immunodeficiency syndrome (AIDS) reported to the Centers for Disease Control (CDC).[1-4] Investigations of 22 of these cases identified at least one donor who was infected with human T-cell lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV), the cause of AIDS.[5] The documentation of asymptomatic HTLV-III/LAV infection in these donors, often years after their donation, underscored the need for a reliable serologic screening procedure to supplement existing self-deferral recommendations.[1,5,6]

Enzyme immunoassays (EIAs) to detect antibody to HTLV-III were developed in 1984 and became commercially available in March 1985 for screening donated blood and plasma.[7-9] The tests had demonstrated high sensitivity and specificity in limited trials before licensure,[8,10-15] but information was not available to assess the significance of positive test results in blood donors, a population with a low prevalence of HTLV-III/LAV infection. The absence of this information created concern that a large number of donated units might be falsely positive, resulting in many donations being needlessly discarded and donors being notified of these false-positive results.[16,17]

After licensure of the first anti-HTLV-III EIA test kit, the CDC and the American Red Cross Blood Services (ARCBS), Atlanta Region, established a cooperative study to evaluate the specificity and predictive value of the antibody test in a blood bank screening program.

## SUBJECTS AND METHODS

### Blood Donor Study

From March 25 through July 31, 1985, all potential donors at the ARCBS continued to be provided with information about self-deferral of donors with a risk for HTLV-III/LAV infection or AIDS and were advised about the availability of alternative tests sites where the anti-HTLV-III test could be obtained inexpensively and anonymously. In addition, as part of the consent procedure for donation, donors were informed that their blood would be tested for antibody to HTLV-III, that additional tests might be performed at the CDC to evaluate test performance, and that they would be contacted if any of the tests suggested they had been exposed to HTLV-III/LAV.

From the AIDS Program, Center for Infectious Diseases, Centers for Disease Control (Drs Ward, Feorino, and Allen and Messrs Schable and Parvin), the American Red Cross Blood Services, Atlanta Region (Dr Grindon), and the Department of Pathology and Laboratory Medicine, Emory University (Dr Grindon), Atlanta.

Reprint requests to Blood and Transfusion Studies, AIDS Branch, Centers for Disease Control, Atlanta, GA 30333 (Dr Ward).



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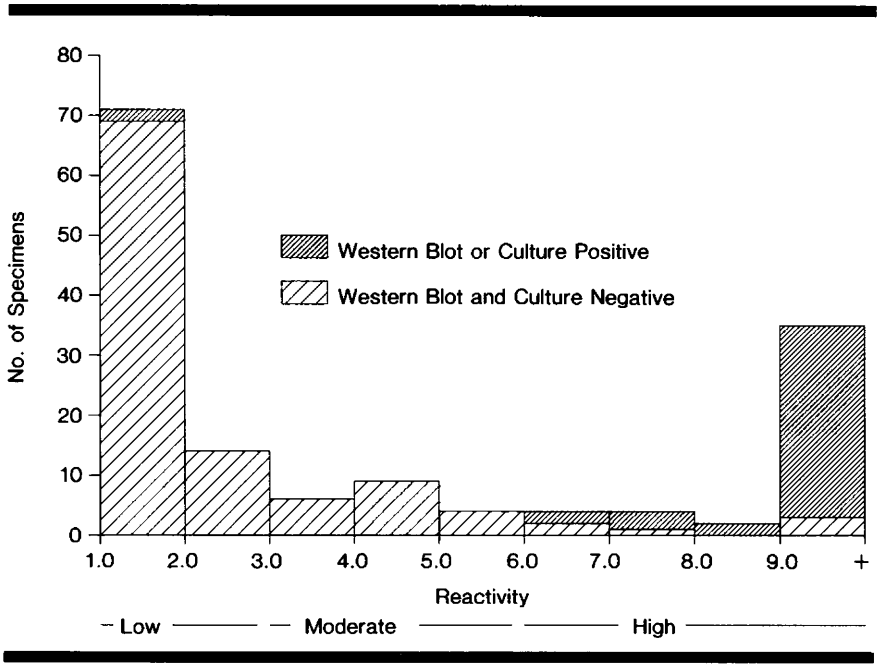
At the time of donation, an additional 20 mL of sterile heparinized blood and 7 mL of clotted blood were collected for the study and stored at room temperature at the ARCBS. Those specimens to be studied were transferred to the CDC within 48 hours after donation; the remainder were discarded.

All units of blood collected in the ARCBS region were processed at a central facility and screened in a routine manner for antibody to HTLV-III, using an EIA. Tests were conducted as recommended by the package insert. Units with an initially negative EIA reaction were not retested at the ARCBS, but specimens from ten of these units each day were selected by systematic random sample and sent to the CDC as controls. Units reactive on the initial EIA test at the ARCBS were tested two additional times. If both subsequent tests were nonreactive, the EIA reaction was interpreted as negative. Units reactive on one or both subsequent EIA tests were interpreted as positive. Specimens from all units initially reactive on EIA irrespective of subsequent results at the ARCBS were sent to the CDC for additional testing. Specimens from approximately 12% of the donors with a positive EIA reaction and from 21% of the control donors were not available for testing at the CDC if the unit was donated on a weekend or if technical difficulties developed in obtaining the specimens from the donor.

Laboratory testing at the CDC included the Western blot assay, using disrupted whole-virus antigen in a microgel assay system with molecular-weight standards and positive and negative controls,[18] and cocultivation of peripheral blood lymphocytes for isolation of HTLV-III/LAV.[3,5] The Western blot assay results were reported as positive if either the p24 and/or gp41 precipitation bands were identified. The Western blot was repeated on specimens with only a p24 or gp41 band, and results were reported as positive only if the single band was consistently identified. Lymphocyte cultures for HTLV-III/LAV were reported as positive on the basis of elevated reverse transcriptase levels and electron microscopy examination. Cultures were not done on approximately 11% of the specimens sent to the CDC because the lymphocytes were not viable by the time the specimen was received. Laboratory personnel conducting the various tests were blinded to results of tests performed at the ARCBS and to other tests performed at the CDC.

Reactivity of the anti-HTLV-III EIA-positive specimens was assessed semiquantitatively by determining a ratio of the test absorbance value to the cutoff value obtained, as outlined in the package insert. The mean of the ratios for all tests performed at the ARCBS for each EIA-positive specimen was calculated and used as the basis for determining the level of reactivity. Categories for classifying the positive EIA results were arbitrarily defined by inspecting the distribution of the EIA mean ratios and distribution of the Western blot and HTLV-III/LAV lymphocyte culture results. The upper limits of the EIA ratios were dictated by the maximum absorbance read by the spectrophotometer, and the variable cutoff value was calculated for each test run. Three categories of EIA test reactivity were defined: low, with a mean ratio from 1.0 to 2.9; moderate, with a mean ratio from 3.0 to 5.9; and high, with a mean ratio of 6.0 or greater.




Distribution of reactivity for specimens found to be positive for human T-cell lymphotropic virus type III on enzyme immunoassays at American Red Cross Blood Services, Atlanta Region, and evaluated at Centers for Disease Control.

### Statistical Methods

Tests for statistical significance included the Spearman rank correlation for testing associations between ordinal variables, the nonparametric Wilcoxon rank-sum test for comparing the median values of two distributions, the Student $t$ test for comparing the mean values of two distributions, and the $\chi^2$ test for comparing two proportions. Fisher's exact test was used instead of the $\chi^2$ test when expected frequencies were small. Exact confidence bounds for proportions were calculated by the Clopper-Pearson method.[20]

### Epidemiologic Investigation

Blood donors were notified of test results by the ARCBS if the EIA reaction was positive or if other test results at the CDC indicated possible HTLV-III/LAV infection. The donor was told about the CDC follow-up study by Red Cross personnel as part of the notification procedure. Those who gave informed consent were interviewed by a CDC investigator to identify risks for HTLV-III/LAV infection. A sample of donors from among those with only an initially reactive EIA and from those with specimens selected as controls were also interviewed. The CDC investigator was blinded to the donors' test results until after completion of the interview.

## RESULTS

### Blood Donor Study

During the 18-week study period, 67 190 units of blood were collected by the ARCBS. The HTLV-III EIA test was initially reactive in 569 (0.85%) of the units, but was repeatedly reactive (positive) in only 171 units (0.25% of all donations). The mean number of EIA-positive specimens per week was 9.5, but this varied widely (range, two to 22). No significant change was found in the rate of EIA-positive specimens as the study progressed ($P>.15$, Spearman's rank correlation).

Specimens from 150 positive units



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Table 1.—Results of Western Blot Assay and HTLV-III* Culture of Specimens Found to Be HTLV-III EIA†-Positive at American Red Cross Blood Services, Atlanta Region

| EIA Reactivity | Western Blot Results: Positive | Western Blot Results: Negative | Total (%) |
|---|---|---|---|
| Low | | | |
| Culture positive | 1 | 1 | 2 (2.3) |
| Culture negative | 0 | 78 | 78 (90.7) |
| Culture not done/contaminated | 0 | 6 | 6 (7.0) |
| **Total (%)** | **1 (1.2)** | **85 (98.8)** | **86 (100.0)** |
| Moderate | | | |
| Culture positive | 0 | 0 | 0 (0.0) |
| Culture negative | 0 | 14 | 14 (73.3) |
| Culture not done/contaminated | 0 | 5 | 5 (26.3) |
| **Total** | **0 (0.0)** | **19 (100.0)** | **19 (100.0)** |
| High | | | |
| Culture positive | 22 | 1 | 23 (51.1) |
| Culture negative | 13 | 4 | 17 (37.8) |
| Culture not done/contaminated | 3 | 2 | 5 (11.1) |
| **Total** | **38 (84.4)** | **7 (15.6)** | **45 (100.0)** |

*HTLV-III indicates human T-cell lymphotropic virus type III.
†EIA indicates enzyme immunoassay.

Table 2.—Comparison of Blood Donors by Sex, Age, and Donation History

| EIA* Reactivity (Ratio) | N | Men: % | Men: Mean Age, y | Women: % | Women: Mean Age, y | No. (%†) Who Donated Blood After March 1983 |
|---|---|---|---|---|---|---|
| Negative (<1.0) | 624‡ | 58 | 31.7 | 42 | 29.4 | 367 (71.4) |
| Low/moderate (1.0-5.9) | 95 | 46 | 37.4 | 54 | 37.0 | 52 (88.0) |
| High (≥6.0) | 43 | 88 | 29.1 | 12 | 32.6 | 20 (83.3) |

*EIA indicates enzyme immunoassay.
†Of those with complete blood donation history on file with American Red Cross Blood Services, Atlanta Region.
‡Record search for vital statistics completed by American Red Cross Blood Services, Atlanta Region.

(87.7%) were sent to the CDC for additional testing; only these specimens are included in our analysis. The distribution of EIA-positive units by degree of test reactivity showed that over half clustered just above the cutoff point (ratio of test absorbence to cutoff value, <3.0). The number of positive blood units with any given reactivity ratio above this value decreased markedly, but the value of the ratios ranged up to 24.0. When test results were categorized by degree of reactivity, they showed a bimodal distribution: 57.3% of the specimens had low reactivity, 12.7% had moderate reactivity, and 30.0% had high reactivity (Figure).

Results of Western blot assay of the EIA-positive specimens correlated highly with degree of EIA test reactivity. Thirty-nine specimens (26.0%) had a positive Western blot result; 38 (84.4%) of the 45 specimens highly reactive on EIA were Western blot positive (Table 1). Only one (1.2%) of the 86 low reactive specimens and none of the 19 moderately reactive specimens had a positive Western blot result ($P<.00001$, Fisher's exact test). Of the Western blot-positive specimens, both the p24 and gp41 bands were present in 28 specimens (71.8%); the p24 band was present alone in 11 specimens (28.2%). The gp41 band did not occur as an isolated finding in any of these specimens.

Lymphocyte cultures for HTLV-III/LAV were completed on 134 (89.3%) of the EIA-positive specimens; HTLV-III/LAV was isolated from 25 (17.2%) of the 134 specimens that could be cultured (Table 1). Specimens highly reactive on EIA were most likely to be culture positive, with HTLV-III/LAV isolated from 23 (57.5%) of 40 cultured specimens in this group. In contrast, none of 14 specimens with moderate EIA reactivity and only two (2.5%) of 80 specimens with low EIA reactivity were culture positive ($P<.0001$, Fisher's exact test).

The Western blot was positive in 23 (92.0%) of the 25 culture-positive specimens (Table 1). Conversely, 23 (63.9%) of the 36 Western blot-positive specimens cultured for HTLV-III/LAV were positive. Virus was isolated from 55.5% of the cultured specimens with only a p24 band on Western blot assay and from 62.9% of those cultured with both p24 and gp41 bands ($P$ was not significant; Fisher's exact test).

Specimens from 628 EIA-negative control donors and from 306 donors whose specimens were initially EIA reactive but subsequently nonreactive on testing at the ARCBS also were evaluated at the CDC. The Western blot assay gave negative results on all specimens in the EIA-negative group, but gave positive results with a p24 band from one specimen that was initially reactive on EIA. Repeated Western blot testing of the same specimen and of a separate specimen obtained at a later date gave negative results when performed by laboratory personnel blinded to the results of the initial test. Lymphocytes from 50 of the seronegative specimens and from 227 of those that were EIA reactive only on the initial test were cultured for HTLV-III/LAV; all results were negative.

Of the 150 EIA-positive specimens from blood donors tested at the CDC, 40 (26.7%) had a Western blot or culture positive for HTLV-III/LAV. If we assume that these are the only true-positives, no more than 125 of the 67 190 units tested were falsely positive. This gives a calculated test specificity of 99.82%.

## Donor Investigation

Demographic characteristics of donors with positive EIA reactions varied by degree of test reactivity and were different from those with negative EIA reactions (Table 2). Donors with low or moderately reactive tests were significantly more likely to be older ($P<.0001$, Student's $t$ test) and female ($P<.05$, $\chi^2$). Donors of highly reactive specimens were almost exclusively male ($P<.0001$, $\chi^2$), with an age distribution similar to that of the seronegative controls. The proportion of donors who had EIA-positive reactions and a history of blood donation since March 31, 1983, was not significantly different than for the seronegative control group ($\chi^2$ test) (Table 2).

Interviews have been completed with 104 (69.3%) of the 150 EIA-positive donors. Twenty-six of these donors had a risk factor for HTLV-III/LAV infection; all but one had highly reactive



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Table 3.—Result of Donor Interviews to Identify Risk Factors for HTLV-III* Infection

| EIA† Reactivity (Ratio) | Risk Identified | | No Risk Identified | |
|---|---|---|---|---|
| | Culture Positive | Culture Negative | Culture Positive | Culture Negative |
| Low (1.0-2.9) | 1 | 0 | 1 | 59 |
| Moderate (3.0-5.9) | 0 | 0 | 0 | 14 |
| High (≥6.0) | 20 | 5 | 2 | 2 |

*HTLV-III indicates human T-cell lymphotropic virus type III.
†EIA indicates enzyme immunoassay.

EIA tests (Table 3). Twenty-four were men who were homosexual or bisexual during the five years before donation. The remaining man and the only woman had been heterosexual partners of an intravenous drug user and a bisexual man, respectively.

Seventy-eight of the 104 donors who were interviewed had no identified risk factor for HTLV-III/LAV infection. Only four of these 78 donors had highly reactive specimens on EIA; two of the four were Western blot and HTLV-III/LAV culture positive. Seventy-four of the 78 donors without an identified risk factor had moderate or low test reactivity on EIA; one of these was a woman who had a negative Western blot reaction but HTLV-III/LAV isolated on culture.

## COMMENT

The anti-HTLV-III EIA test was successfully implemented into the routine screening procedure of the voluntary blood collection program at the ARCBS. The use of the test resulted in only 0.25% of donations being withheld from transfusion. The proportion of HTLV-III antibody-positive donors at the ARCBS is consistent with findings of a national survey conducted by the Food and Drug Administration.[21]

Evaluation of a new test requires an established or known standard for comparison. At this point, however, no established standard exists for identifying HTLV-III infection in asymptomatic people. Current culture methods for HTLV-III identify virus in only 36% to 85% of persons with AIDS or related conditions[22] and cannot be used as an absolute standard for HTLV-III/LAV infection. For this reason, we defined specimens positive on Western blot or culture as positive for infection with HTLV-III/LAV.

In a population with a defined prevalence of infection, positive predictive value for test results varies depending on the sensitivity and specificity of each test; for a given test with an established sensitivity and specificity, positive predictive value will vary with the prevalence of infection in the populations studied. In blood donors in our study, the proportion of all EIA-positive specimens infected with HTLV-III/LAV, or the positive predictive value, was 27.3%. The positive predictive value for the EIA test increased to 86.3% (95% confidence limits, 73.2% to 95.5%; binomial distribution) if the mean EIA ratio was 6.0 or greater. Conversely, the predictive value for test specimens with a mean EIA ratio of 1.0 to 5.9 was only 1.9% (95% confidence limits, 0.02% to 5.2%; binomial distribution).

Donors of EIA-positive specimens negative on Western blot and culture were more likely to be older and female than were seronegative donors. Others have found a similar relationship.[23] Antibody to the HLA-DR4 or other cellular antigens may be the cause of some false-positive reactions.[24]

Only the p24 band was identified in 28.2% of blood donors with a positive Western blot reaction. The presence of only a single band was verified by repeated testing and did not significantly affect the ability to culture virus. Persons recently infected with HTLV-III/LAV have been found on Western blot to initially have antibodies to p24 alone, followed by the later development of antibodies to gp41.[25,26]

Our findings indicate that the anti-HTLV-III EIA is specific. We calculated the test specificity in this study to be 99.82%, which is almost identical with the 99.8% specificity estimated from studies conducted before licensure of the test.[27]

Of 628 anti-HTLV-III EIA control specimens tested by Western blot and culture, none were positive for HTLV-III/LAV infection. The size and the low prevalence of HTLV-III/LAV infection of the control group do not allow for an accurate estimate of sensitivity. A study of similar design in a group of homosexual men at high risk for HTLV-III/LAV infection found none of 70 HTLV-III EIA-negative specimens positive on viral culture, while 43 (60%) of 72 EIA-positive specimens were culture positive.[28] Trials of test performance in symptomatic HTLV-III/LAV-infected persons conducted before licensure obtained a sensitivity of 95%[27]; as persons with AIDS may lose antibody, however, this may represent a low estimate.[8] In selected populations, a small number of seronegative persons without AIDS have been found positive on HTLV-III/LAV culture.[29,30]

Our study supports the need to discard units of blood that have a positive EIA reaction, as defined by the test manufacturer. Although we found no units of blood that were Western blot or culture positive if they had only an initially reactive (nonrepeatable) EIA test, we had 2 units that had a positive EIA reaction and a negative Western blot result that were culture positive for HTLV-III/LAV. It is therefore important for persons at risk of infection to continue to refrain from donating blood.

Our study found little evidence that persons were donating blood primarily to have their antibody status determined. Most of the donors who had Western blot or HTLV-III/LAV culture-positive specimens had donated previously, and the proportion who were new donors was not different from those who were seronegative.

The study identified 26 HTLV-III/LAV-infected persons who donated blood after being provided with recommendations for self-deferral of donors with risks for HTLV-III/LAV infection. The reason why these persons continued to donate blood is unclear, although anecdotal information obtained on interview indicated that some donors did not perceive themselves at risk for HTLV-III/LAV infection. The recommendations for donor deferral have been reworded to better inform persons of the reason for not donating blood.[31]

The number of units of blood donated by HTLV-III/LAV-infected people is a small fraction of all donations, but the HTLV-III EIA screening program should have significant impact on potential virus transmission to transfusion recipients. The ARCBS collects more than 194,000 units of blood each year in Georgia, and an average of two blood components are derived from each unit. If the 0.07% prevalence rate of HTLV-III/LAV infection among blood donors remains constant, an estimated 136 seropositive units will be identified and 272 HTLV-III/LAV infections among transfusion recipients potentially averted in the Atlanta Region alone.

New cases of transfusion-associated AIDS will continue to occur among people exposed before the screening program was initiated, but the current



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use of the EIA test to screen donated blood will markedly reduce the risk of exposure by this route in the future and ultimately the number of transfusion-associated AIDS cases.

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# ATTACHMENT 11

Weekly
May 23, 1986 / 35(20);334-9

# Current Trends Classification System for Human T-Lymphotropic Virus Type III/ Lymphadenopathy-Associated Virus Infections

## INTRODUCTION

Persons infected with the etiologic retrovirus of acquired immunodefi- ciency syndrome (AIDS) (1-4) * may present with a variety of manifestations ranging from asymptomatic infection to severe immunodeficiency and life- threatening secondary infectious diseases or cancers. The rapid growth of knowledge about human T-lymphotropic virus type III/lymphadenopathy- associated virus (HTLV-III/LAV) has resulted in an increasing need for a system of classifying patients within this spectrum of clinical and laboratory findings attributable to HTLV-III/LAV infection (5-7).

Various means are now used to describe and assess patients with manifes- tations of HTLV-III/LAV infection and to describe their signs, symptoms, and laboratory findings. The surveillance definition of AIDS has proven to be extremely valuable and quite reliable for some epidemiologic studies and clinical assessment of patients with the more severe manifestations of disease. However, more inclusive definitions and classifications of HTLV- III/LAV infection are needed for optimum patient care, health planning, and public health control strategies, as well as for epidemiologic studies and special surveys. A broadly applicable, easily understood classification system should also facilitate and clarify communication about this disease.

In an attempt to formulate the most appropriate classification system, CDC has sought the advice of a panel of expert consultants ** to assist in defining the manifestations of HTLV-III/LAV infection.

## GOALS AND OBJECTIVES OF THE CLASSIFICATION SYSTEM

The classification system presented in this report is primarily applicable to public health purposes, including disease reporting and surveillance, epidemiologic studies, prevention and control activities, and public health policy and planning.

Immediate applications of such a system include the classification of infected persons for reporting of cases to state and local public health agencies, and use in various disease coding and recording systems, such as the forthcoming 10th revision of the International Classification of Diseases.

## DEFINITION OF HTLV-III/LAV INFECTION

The most specific diagnosis of HTLV-III/LAV infection is by direct identification of the virus in host tissues by virus isolation; however, the techniques for isolating HTLV-III/LAV currently lack sensitivity for detecting infection and are not readily available. For public health purposes, patients with repeatedly reactive screening tests for HTLV-III/LAV antibody (e.g., enzyme-linked immunosorbent assay) in whom antibody is also identified by the use of supplemental tests (e.g., Western blot, immunoflu- orescence assay) should be considered both infected and infective (8-10). Although HTLV-III/LAV infection is identified by isolation of

the virus or, indirectly, by the presence of antibody to the virus, a presumptive clinical diagnosis of HTLV-III/LAV infection has been made in some situations in the absence of positive virologic or serologic test results. There is a very strong correlation between the clinical manifestations of AIDS as defined by CDC and the presence of HTLV-III/LAV antibody (11-14). Most persons whose clinical illness fulfills the CDC surveillance definition for AIDS will have been infected with the virus (12-14).

## CLASSIFICATION SYSTEM

This system classifies the manifestations of HTLV-III/LAV infection into four mutually exclusive groups, designated by Roman numerals I through IV (Table 5). The classification system applies only to patients diagnosed as having HTLV-III/LAV infection (see previous section, DEFINITION OF HTLV- III/LAV INFECTION). Classification in a particular group is not explicitly intended to have prognostic significance, nor to designate severity of illness. However, classification in the four principal groups, I-IV, is hierarchical in that persons classified in a particular group should not be reclassified in a preceding group if clinical findings resolve, since clinical improvement may not accurately reflect changes in the severity of the underlying disease.

Group I includes patients with transient signs and symptoms that appear at the time of, or shortly after, initial infection with HTLV-III/LAV as identified by laboratory studies. All patients in Group I will be reclassified in another group following resolution of this acute syndrome.

Group II includes patients who have no signs or symptoms of HTLV-III/LAV infection. Patients in this category may be subclassified based on whether hematologic and/or immunologic laboratory studies have been done and whether results are abnormal in a manner consistent with the effects of HTLV-III/LAV infection.

Group III includes patients with persistent generalized lymphadenopathy, but without findings that would lead to classification in Group IV. Patients in this category may be subclassified based on the results of laboratory studies in the same manner as patients in Group II.

Group IV includes patients with clinical symptoms and signs of HTLV- III/LAV infection other than or in addition to lymphadenopathy. Patients in this group are assigned to one or more subgroups based on clinical findings. These subgroups are: A. constitutional disease; B. neurologic disease; C. secondary infectious diseases; D. secondary cancers; and E. other conditions resulting from HTLV-III/LAV infection. There is no a priori hierarchy of severity among subgroups A through E, and these subgroups are not mutually exclusive.

Definitions of the groups and subgroups are as follows:

Group I. Acute HTLV-III/LAV Infection. Defined as a mononucleosis-like syndrome, with or without aseptic meningitis, associated with seroconversion for HTLV-III/LAV antibody (15-16). Antibody seroconversion is required as evidence of initial infection; current viral isolation procedures are not adequately sensitive to be relied on for demonstrating the onset of infection.

Group II. Asymptomatic HTLV-III/LAV Infection. Defined as the absence of signs or symptoms of HTLV-III/LAV infection. To be classified in Group II, patients must have had no previous signs or symptoms that would have led to classification in Groups III or IV. Patients whose clinical findings caused them to be classified in Groups III or IV should not be reclassified in Group II if those clinical findings resolve.

Patients in this group may be subclassified on the basis of a laboratory evaluation. Laboratory studies commonly indicated for patients with HTLV- III/LAV infection include, but are not limited to, a complete blood count (including differential white blood cell count) and a platelet count. Immuno- logic tests, especially T-lymphocyte helper and suppressor cell counts, are also an important part of the overall evaluation. Patients whose test results are within normal limits, as well as those for whom a laboratory evaluation has not yet been completed, should be differentiated from patients whose test results are consistent with defects associated with HTLV-III/LAV infection (e.g., lymphopenia, thrombocytopenia, decreased number of helper (T((4))) T-lymphocytes).

Group III. Persistent Generalized Lymphadenopathy (PGL). Defined as palpable lymphadenopathy (lymph node enlargement of 1 cm or greater) at two or more extra-inguinal sites persisting for more than 3 months in the absence of a concurrent illness or condition other than HTLV-III/LAV infection to explain the findings. Patients in this group may also be subclassified on the basis of a laboratory evaluation, as is done for asymptomatic patients in Group II (see above). Patients with PGL whose clinical findings caused them to be classified in Group IV should not be reclassified in Group III if those other clinical findings resolve.

Group IV. Other HTLV-III/LAV Disease. The clinical manifestations of patients in this group may be designated by assignment to one or more subgroups (A-E) listed below. Within Group IV, subgroup classification is independent of the presence or absence of lymphadenopathy. Each subgroup may include patients who are minimally symptomatic, as well as patients who are severely ill. Increased specificity for manifestations of HTLV-III/LAV infection, if needed for clinical purposes or research purposes or for disability determinations, may be achieved by creating additional divisions within each subgroup.

Subgroup A. Constitutional disease. Defined as one or more of the following: fever persisting more than 1 month, involuntary weight loss of greater than 10% of baseline, or diarrhea persisting more than 1 month; and the absence of a concurrent illness or condition other than HTLV- III/LAV infection to explain the findings.

Subgroup B. Neurologic disease. Defined as one or more of the following: dementia, myelopathy, or peripheral neuropathy; and the absence of a concurrent illness or condition other than HTLV-III/LAV infection to explain the findings.

Subgroup C. Secondary infectious diseases. Defined as the diagnosis of an infectious disease associated with HTLV-III/LAV infection and/or at least moderately indicative of a defect in cell-mediated immunity. Patients in this subgroup are divided further into two categories:

Category C-1. Includes patients with symptomatic or invasive disease due to one of 12 specified secondary infectious diseases listed in the surveillance definition of AIDS ***: Pneumocystis carinii pneumonia, chronic cryptosporidiosis, toxoplasmosis, extra- intestinal strongyloidiasis, isosporiasis, candidiasis (esophageal, bronchial, or pulmonary), cryptococcosis, histoplasmosis, mycobacterial infection with Mycobacterium avium complex or M. kansasii, cytomegalovirus infection, chronic mucocutaneous or disseminated herpes simplex virus infection, and progressive multifocal leukoencephalopathy.

Category C-2. Includes patients with symptomatic or invasive disease due to one of six other specified secondary infectious diseases: oral hairy leukoplakia, multidermatomal herpes zoster, recurrent Salmonella bacteremia, nocardiosis, tuberculosis, or oral candidiasis (thrush).

Subgroup D. Secondary cancers. Defined as the diagnosis of one or more kinds of cancer known to be associated with HTLV-III/LAV infection as listed in the surveillance definition of AIDS and at least moderately indicative of a defect in cell-mediated immunity ****: Kaposi's sarcoma, non-Hodgkin's lymphoma (small, noncleaved lymphoma or immunoblastic sarcoma), or primary lymphoma of the brain.

Subgroup E. Other conditions in HTLV-III/LAV infection. Defined as the presence of other clinical findings or diseases, not classifiable above, that may be attributed to HTLV-III/LAV infection and/or may be indicative of a defect in cell-mediated immunity. Included are patients with chronic lymphoid interstitial pneumonitis. Also included are those patients whose signs or symptoms could be attributed either to HTLV-III/LAV infection or to another coexisting disease not classified elsewhere, and patients with other clinical illnesses, the course or management of which may be comp- licated or altered by HTLV-III/LAV infection. Examples include: patients with constitutional symptoms not meeting the criteria for sub-group IV-A; patients with infectious diseases not listed in subgroup IV-C; and patients with neoplasms not listed in subgroup IV-D.

Reported by Center for Infectious Diseases, CDC.

## Editorial Note

Editorial Note: The classification system is meant to provide a means of grouping patients infected with HTLV-III/LAV according to the clinical expression of disease. It will require periodic revision as warranted by new information about HTLV-III/LAV infection. The definition of particular syndromes will evolve with increasing knowledge of the significance of certain clinical findings and laboratory tests. New diagnostic techniques, such as the detection of specific HTLV-III/LAV antigens or antibodies, may add specificity to the assessment of patients infected with HTLV-III/LAV.

The classification system defines a limited number of specified clinical presentations. Patients whose signs and symptoms do not meet the criteria for other groups and subgroups, but whose findings are attributable to HTLV-III/LAV infection, should be classified in subgroup IV-E. As the classifi- cation system is revised and updated, certain subsets of patients in subgroup IV-E may be identified as having related groups of clinical findings that should be separately classified as distinct syndromes. This could be accom- plished either by creating additional subgroups within Group IV or by broadening the definitions of the existing subgroups.

Persons currently using other classification systems (6-7) or nomen- clatures (e.g., AIDS-related complex, lymphadenopathy syndrome) can find equivalences with those systems and terminologies and the classification presented in this report. Because this classification system has only four principal groups based on chronology, presence or absence of signs and symptoms, and the type of clinical findings present, comparisons with other classifications based either on clinical findings or on laboratory assessment are easily accomplished.

This classification system does not imply any change in the definition of AIDS used by CDC since 1981 for national reporting. Patients whose clinical presentations fulfill the surveillance definition of AIDS are classified in Group IV. However. not every case in Group IV will meet the surveillance definition.

Persons wishing to comment on this material are encouraged to send comments in writing to the AIDS Program, Center for Infectious Diseases, CDC.

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The AIDS virus has been variously termed human T-lymphotropic virus type III (HTLV-III), lymphadenopathy-associated virus (LAV), AIDS-associated retrovirus (ARV), or human immunodeficiency virus (HIV). The designation human immunodeficiency virus (HIV) has recently been proposed by a subcom- mittee of the International Committee for the Taxonomy of Viruses as the appropriate name for the retrovirus that has been implicated as the causative agent of AIDS (4).

** The following persons served on the review panel: DS Burke, MD, RR Redfield, MD, Walter Reed Army Institute of Research, Washington, DC; J Chin, MD, State Epidemiologist, California Department of Health Services; LZ Cooper, MD, St Luke's-Roosevelt Hospital Center, New York City; JP Davis, MD, State Epidemiologist, Wisconsin Division of Health; MA Fischl, MD, University of Miami School of Medicine, Miami, Florida; G Friedland, MD, Albert Einstein College of Medicine, New York City; MA Johnson, MD, DI Abrams, MD, San Francisco General Hospital; D Mildvan, MD, Beth Israel Medical Center, New York City; CU Tuazon, MD, George Washington University School of Medicine, Washington, DC; RW Price, MD, Memorial Sloan-Kettering Cancer Center, New York City; C Konigsberg, MD, Broward County Public Health Unit, Fort Lauderdale, Florida; MS Gottlieb, MD, University of California -- Los Angeles Medical Center; representatives of the National Institute of Allergy and Infectious Diseases, National Cancer Institute, National Institutes of Health, Center for Infectious Diseases, CDC.

*** This subgroup includes patients with one of more of the specified infectious diseases listed whose clinical presentation fulfills the definition of AIDS as used by CDC for national reporting.

**** This subgroup includes those patients with one or more of the specified cancers listed whose clinical presentation fulfills the definition of AIDS as used by CDC for national reporting.

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# ATTACHMENT 12

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MMWR Weekly

July 13, 1984 / 33(27);377-9

# Antibodies to a Retrovirus Etiologically Associated with Acquired Immunodeficiency Syndrome (AIDS) in Populations with Increased Incidences of the Syndrome

Evidence implicates a retrovirus as the etiologic agent of acquired immunodeficiency syndrome (AIDS). Two prototype isolates have been described. One was isolated from the lymph node cells of a homosexual man with unexplained generalized lymphadenopathy, a syndrome associated with AIDS, and was termed lymphadenopathy-associated virus (LAV) (1). A morphologically similar T-lymphotropic retrovirus (HTLV-III) was isolated from lymphocytes of 26 (36%) of 72 patients with AIDS and from 18 (86%) of 21 patients with conditions thought to be related to AIDS (2). The isolation of retroviruses antigenically identical to LAV from a blood donor-recipient pair, each of whom developed AIDS, provides further evidence that this virus is the etiologic agent of AIDS and may be transmitted through blood transfusion (3).

Although direct comparative results have not been published, HTLV-III and LAV are likely to be the same virus because: they have the same appearance by electron microscopy; they are both lymphotropic and cytopathic for OKT-4 cells; isolates from American AIDS patients, when compared, were immunologically indistinguishable from LAV (3); serologic tests of a large number of specimens from patients with AIDS or related conditions show similar results when either of the prototype viruses is used as antigen (4); and preliminary results suggest that LAV and HTLV-III are at least highly related based on competitive radioimmunoassay of their core proteins (5).

Three basic serologic procedures are currently described for detection of antibody to HTLV-III/LAV: an enzyme-linked immunosorbent assay (ELISA) to whole disrupted virus (6-8); a radioimmunoprecipitation assay (RIPA) to the presumed major core protein (called p25) of LAV (9); and assay of antibody to major viral antigens by the Western blot technique (10, 11). Sera from several high-risk populations are being tested by these techniques by the National Cancer Institute, the Institut Pasteur, and CDC, with the support of numerous collaborators. The objectives of these investigations are to determine the frequency of exposure to HTLV-III/LAV and to correlate seropositivity with current infection, clinical signs and symptoms, and prognosis.

Preliminary data suggest that serologic evidence of exposure to HTLV-III/LAV may be common in certain populations at increased risk for AIDS. Antibody to HTLV-III was detected by ELISA in sera from six (35%) of 17 American homosexual men without symptoms of AIDS (6). Sera from eight (18%) of 44 homosexual men without lymphadenopathy attending a venereal disease clinic in Paris had antibody detected by ELISA to LAV (7). Antibody prevalence to LAV (RIPA) has increased from 1% (1/100) in 1978 to 25% (12/48) in 1980 and 65% (140/215) in 1984 among samples of sera from homosexual men attending a sexually transmitted diseases clinic in San Francisco (12). Antibody prevalence among the above men tested in 1984 who had no symptoms or clinical signs of AIDS or related conditions was 55% (69/126) (12). In New York City, where the AIDS cases among intravenous (IV) drug users are concentrated, 87% (75/86) of recent heavy IV drug users without AIDS had antibody to LAV by ELISA, while over 58% (50/86) of the same group had antibody to LAV

detected by RIPA (13). In contrast, fewer than 10% of 35 methadone patients from New York City had antibody to LAV detected by RIPA. All of these latter patients had been in treatment at least 3 years with greatly reduced IV drug usage (14). Seventy-two percent (18/25) of asymptomatic persons with hemophilia A in a home-care treatment program demonstrated antibody to LAV antigens utilizing the Western blot technique (11). All had used factor VIII concentrates from 1980 to 1982. Reported by DC Des Jarlais, PhD, New York State Div of Substance Abuse Svcs, M Marmor, PhD, H Cohen, MPH, New York University Medical Center, S Yancovitz, MD, J Garber, Beth Israel Medical Center, S Friedman, PhD, Narcotic and Drug Research, MJ Kreek, MD, A Miescher, MD, E Khuri, MD, Rockefeller University, New York City, SM Friedman, MD, New York City Dept of Health, R Rothenberg, MD, State Epidemiologist, New York State Dept of Health; D Echenberg, MD, P O'Malley, E Braff, MD, San Francisco City/County Health Dept, J Chin, MD, State Epidemiologist, California Dept of Health Svcs; P Burtenol, MD, Hemophilia of Georgia, Atlanta, RK Sikes, DVM, State Epidemiologist, Georgia Dept of Human Resources; Div of Viral Diseases, Div of Host Factors, AIDS Activity, Center for Infectious Diseases, CDC.

## Editorial Note

Editorial Note: The high prevalence of antibody to HTLV-III/LAV among these groups and the increasing prevalence among homosexual men in San Francisco add further support to HTLV-III/LAV being the etiologic agent of AIDS. They further demonstrate that exposure to the virus is much more common than AIDS itself among populations with increased incidences of the disease. If AIDS follows the pattern of many other infectious diseases, host response to infection would be expected to range from subclinical to severe. Milder disease states for AIDS have been suspected, since the reported frequency of lymphadenopathy and immunologic abnormalities, conditions associated with AIDS, has also been high in these groups. These data, based on limited samples of high-risk groups, suggest the spectrum of response to infection with HTLV-III/LAV may be wide.

These serologic tests are sufficiently sensitive and specific to be of value in estimating the frequency of infection with HTLV-III/LAV in certain populations and for providing important information about the natural history of the disease in such groups. Less clear are the implications of a positive test result for an individual. For some, the result may be a false positive caused by infection with an antigenically related virus or nonspecific test factors. The determination of the frequency and cause of falsely positive tests is essential for proper interpretation of test results, but remains to be established, particularly in populations, such as blood donors who belong to no known AIDS risk groups, where the prevalence of true infection with HTLV-III/LAV is expected to be very low.

A positive test for most individuals in populations at greater risk of acquiring AIDS will probably mean that the individual has been infected at some time with HTLV-III/LAV. Whether the person is currently infected or immune is not known, based on the serologic test alone--HTLV-III/LAV has been isolated in both the presence and absence of antibody--but the frequency of virus in antibody-positive persons is yet to be determined. For seropositive individuals with mild or no signs of disease, including those in whom the virus can be demonstrated, the prognosis remains uncertain. The incubation period for the life-threatening manifestations of AIDS may range from 1 year to more than 4 years (15).

Carefully planned and executed studies will be required to resolve these issues, and to clarify remaining questions about the natural history of AIDS and risk factors for transmission of the virus.

Until the usefulness of positive and negative serologic tests is fully established, all individuals in populations with increased incidences of AIDS, as well as those outside such groups with positive tests, should comply with the March 1983 Public Health Service recommendations for the prevention of AIDS to minimize the transmission of the syndrome (16). Abstention from IV drug usage and reduction of needle-sharing and other use of contaminated needles by IV drug users should also be effective in preventing transmission of the virus and of AIDS. There remains no evidence of transmission of AIDS through casual contact. Prevention measures should stress that transmission has been only through intimate sexual contact, sharing of contaminated needles, or, less frequently, through transfusion of blood or blood products.

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# ATTACHMENT 13

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MMWR Weekly

August 09, 1985 / 34(31);477-8

# Current Trends Update: Public Health Service Workshop on Human T-Lymphotropic Virus Type III Antibody Testing -- United States

The enzyme immunoassay (EIA) serologic tests to detect antibody to human T-lymphotropic virus type III (HTLV-III) are highly sensitive and specific, according to reports presented at a U.S. Public Health Service Workshop on HTLV-III Antibody Testing on July 31, 1985. The tests are currently being used at blood banks, plasma collection centers, health departments, and selected clinical centers throughout the United States.

The U.S. Food and Drug Administration reported cumulative HTLV-III antibody test data from more than 1.1 million units of blood collected at 155 centers through June 16, 1985. Of these, 2,831 (0.25%) were reported as positive based on a repeatedly reactive EIA test. The pattern of positive tests varied slightly in different regions of the country and by test kit used.

The Atlanta Region of the American Red Cross (ARC) and CDC reported data from testing more than 51,000 blood donors, of whom 0.23% were repeatedly reactive by the Abbott EIA method.* Among the specimens from 106 blood donors with repeatedly reactive tests, 34 (32%) were strongly reactive (ratio of specimen absorbance to cutoff value 7.0 or greater). EIA tests categorized as strongly reactive correlated highly with both positive Western blot tests (94%) and culture for HTLV-III/lymphadenopathy-associated virus (LAV) (56%).

Of 220 donors whose tests were initially reactive and subsequently negative, as well as a random sample of 50 with an initially negative EIA test, none had either a positive Western blot test or positive culture. Among those donors notified and interviewed to date, 16 (89%) of 18 with strongly reactive EIA tests had identifiable risk factors for HTLV-III/LAV infection, while none of 20 with weakly reactive tests had identifiable risk factors.

To determine the sensitivity of the Abbott EIA test in high-risk persons, virus isolations were attempted from homosexual men attending a clinic for sexually transmitted diseases in San Francisco, California. None of 70 men with negative HTLV-III antibody tests had a positive culture, while 43 (60%) of 72 with repeatedly reactive tests were culture-positive. Among the 72 EIA-positive sera in this portion of the study, 70 (97%) were considered to be highly reactive. Ninety-seven percent of those EIA-positive specimens tested to date have had a positive Western blot test.

Data from other blood banking organizations paralleled the findings of the ARC/CDC study in suggesting that approximately one-third of EIA-positive sera from blood donors were strongly reactive, regardless of the test kit used. Donors with strongly reactive EIA tests were also highly likely to have positive Western blot tests and to have positive EIA tests by other test kits.

Weakly reactive EIA tests correlated poorly with positive Western blot tests and were judged to be nonspecific for HTLV-III/LAV infection. The reason for nonspecific test reactivity is unknown, but proposed refinements in the test may eliminate many of the low level reactions. Reported by Center for Drugs and Biologics, U.S. Food and Drug Administration; AIDS Br, Div of Viral Diseases, Center for Infectious Diseases, CDC.

## Editorial Note

Editorial Note: Based on available data, only about 0.25% (1 in 400) blood donors have repeatedly reactive EIA tests to HTLV-III antibody. Approximately 0.08% (1 in 1,200) donors were found to have strongly reactive EIA tests, and these donors were likely to have other test results (Western blot, HTLV-III/LAV culture) that suggested they had been infected with HTLV-III/LAV.

Thus, in less than 5 months, serologic tests for HTLV-III antibody have been introduced and demonstrated to be highly useful in screening donated blood. Screening performed during this period may have removed as many as 1,000 potentially infectious units of blood from the U.S. blood supply. Continued use of this highly sensitive test procedure for HTLV-III antibody, in combination with voluntary avoidance of donation by members of high-risk groups, will virtually eliminate the risk of acquired immunodeficiency syndrome (AIDS) transmission by the nation's blood supply. Discussions and evaluations of other potentially appropriate and useful applications of this test are under way. *Use of trade names is for identification only and does not imply endorsement by CDC or the U.S. Public Health Service.

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MMWR

Weekly

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# Provisional Public Health Service Inter-Agency Recommendations for Screening Donated Blood and Plasma for Antibody to the Virus Causing Acquired Immunodeficiency Syndrome

In March 1983, the U.S. Public Health Service issued inter-agency recommendations on the prevention of acquired immunodeficiency syndrome (AIDS) (1). Included was the recommendation that members of groups at increased risk for AIDS should refrain from donating plasma and/or blood. That recommendation was made to decrease the risk of AIDS associated with the administration of blood or blood products, which accounts for about 2% of all reported AIDS cases in the United States.

Evidence has shown that a newly recognized retrovirus is the cause of AIDS. Although this virus has been given several names, including human T- lymphotropic virus type III (HTLV-III) (2), lymphadenopathy-associated virus (LAV) (3), and AIDS-associated retrovirus (ARV) (4), it is referred to as HTLV-III in this discussion. Tests to detect antibody to HTLV-III will be licensed and commercially available in the United States in the near future to screen blood and plasma for laboratory evidence of infection with the virus. The antibody tests are modifications of the enzyme-linked immunosorbent assay (ELISA), which uses antigens derived from whole disrupted HTLV-III (5).

There is considerable experience with the ELISA test in research laboratories, but much additional information will be gathered following its widespread application. In the early phases of testing, a number of false- positive tests may be encountered. Adjustments in interpretation are anticipated as more is learned about the performance of the test in an individual laboratory and about the specific proportion of falsely positive or falsely negative tests in the screening setting where the test is used.

The present recommendations concern the use of these tests to screen blood and plasma collected for transfusion or manufactured into other products. They are intended to supplement, rather than replace, the U.S. Food and Drug Administration's recently revised recommendations to blood and plasma collection facilities and the earlier inter-agency recommendations (1). Additional public health applications of these tests in the under- standing and control of AIDS will be described in a subsequent report.

## BACKGROUND

### Antibody Detection Studies

The ELISA test has been used in many research programs for detecting antibodies to HTLV-III in patients with AIDS and with AIDS-related conditions. In different studies, HTLV-III antibody was found to range from 68% to 100% of patients with AIDS, and in 84%-100% of persons with related conditions, such as unexplained generalized lymphadenopathy (5-7). Serologic surveys have yielded variable seropositivity rates in groups at increased risk for AIDS: 22%-65% of homosexual men (8-11), 87% of intravenous-drug abusers admitted to a

detoxification program in New York City (12), 56%-72% of persons with hemophilia A (13,14), and 35% of women who were sexual partners of men with AIDS (15). In contrast to the above groups, HTLV-III antibody has been detected in fewer than 1% of persons with no known risks for AIDS (4-10).

The time needed to develop a positive antibody test following infection is not known. Data regarding the interval between infection with HTLV-III and seroconversion are limited. A nurse who sustained a needle-stick injury while caring for an AIDS patient developed antibody between 4 and 7 weeks following exposure (16). Additionally, a recent study described several asymptomatic individuals infected with HTLV-III for more than 6 months in the absence of detectable antibody (17,18). Nonetheless, currently available ELISA tests can be expected to identify most persons with HTLV-III infection.

Virus Isolation Studies

HTLV-III has been isolated from blood, semen, and saliva and has been recovered from many individuals in the presence of antibody (19,20). HTLV-III has been isolated from the blood of 85% or more of seropositive individuals with AIDS (21), lymphadenopathy, or other AIDS-associated conditions (2) and from three of four mothers of infants with AIDS (2). The virus has also been isolated from asymptomatic seropositive homosexual men and hemophiliacs, and has been recovered from 95% of seropositive high-risk blood donors who had been implicated in the transmission of AIDS through transfusion (21). The recovery of HTLV-III from these high-risk donors 2 or more years after their initial donation provides evidence that viremia may persist for years in both asymptomatic and symptomatic individuals. HTLV-III has also been isolated from some asymptomatic seronegative persons, but this is the exception (17).

Modes of Transmission

Epidemiologic data suggest that the virus has been transmitted through intimate sexual contact; sharing contaminated needles; transfusion of whole blood, blood cellular components, plasma, or clotting factor concentrates that have not been heat treated; or from infected mother to child before, at, or shortly after the time of birth. No other products prepared from blood (e.g., immunoglobulin, albumin, plasma protein fraction, hepatitis B vaccine) have been implicated, nor have cases been documented to occur through such common exposures as sharing meals, sneezing or coughing, or other casual contact.

Natural History of Infection

Information about the course of infection with HTLV-III is incomplete, but the majority of infected adults will not acquire clinically apparent AIDS in the first few years after infection. In some studies 5%-19% of sero-positive homosexual men developed AIDS within 2-5 years after a previously collected serum sample was retrospectively tested and found to be sero- positive. An additional 25% developed generalized lymphadenopathy, oral candidiasis, or other AIDS-associated conditions within the same interval (11,22). The long-term prognosis for most persons infected with HTLV-III is unknown.

SCREENING BLOOD AND PLASMA

Initial Testing

Persons accepted as donors should be informed that their blood or plasma will be tested for HTLV-III antibody. Persons not wishing to have their blood or plasma tested must refrain from donation. Donors should be told that they will be notified if their test is positive and that they may be placed on the collection facility's donor deferral list, as is currently practiced with other infectious diseases, and should be informed of the identities of additional deferral lists to which the positive donors may be added.

All blood or plasma should be tested for HTLV-III antibody by ELISA. Any blood or plasma that is positive on initial testing must not be transfused or manufactured into other products capable of transmitting infectious agents.

When the ELISA is used to screen populations in whom the prevalence of HTLV-III infections is low, the proportion of positive results that are falsely positive will be high. Therefore, the ELISA should be repeated on all seropositive specimens before the donor is notified. If the repeat ELISA test is negative, the specimen should be tested by another test.

Other Testing

Other tests have included immunofluorescence and radioimmunoprecipitation assays, but the most extensive experience has been with the Western blot technique (22), in which antibodies can be detected to HTLV-III proteins of specific molecular weights. Based on available data, the Western blot should be considered positive for antibody to HTLV-III if band p24 or gp41 is present (alone or in combination with other bands).

Notification of Donors

If the repeat ELISA test is positive or if other tests are positive, it is the responsibility of the collection facility to ensure that the donor is notified. The information should be given to the donor by an individual especially aware of the sensitivities involved. At present, the proportion of these seropositive donors who have been infected with HTLV-III is not known. It is, therefore, important to emphasize to the donor that the positive result is a preliminary finding that may not represent true infection. To determine the significance of a positive test, the donor should be referred to a physician for evaluation. The information should be given to the donor in a manner to ensure confidentiality of the results and of the donor's identify.

Maintaining Confidentiality

Physicians, laboratory and nursing personnel, and others should recognize the importance of maintaining confidentiality of positive test results. Disclosure of this information for purposes other than medical or public health could lead to serious consequences for the individual. Screening procedures should be designed with safeguards to protect against unauthorized disclosure. Donors should be given a clear explanation of how information about them will be handled. Facilities should consider developing contingency plans in the event that disclosure is sought through legal process. If donor deferral lists are kept, it is necessary to maintain confidentiality of such lists. Whenever appropriate, as an additional safeguard, donor deferral lists should be general, without indication of the reason for inclusion.

Medical Evaluation

The evaluation might include ELISA testing of a follow-up serum specimen and Western blot testing, if the specimen is positive. Persons who continue to show serologic evidence of HTLV-III infection should be questioned about possible exposure to the virus or possible risk factors for AIDS in the individual or his/her sexual contacts and examined for signs of AIDS or related conditions, such as lymphadenopathy, oral candidiasis, Kaposi's sarcoma, and unexplained weight loss. Additional laboratory studies might include tests for other sexually transmitted diseases, tests of immune function, and where available, tests for the presence of the virus, such as viral culture. Testing for antibodies to HTLV-III in the individual's sexual contacts may also be useful in establishing whether the test results truly represent infection.

RECOMMENDATIONS FOR THE INDIVIDUAL

An individual judged most likely to have an HTLV-III infection should be provided the following information and advice:

1. The prognosis for an individual infected with HTLV-III over the long term is not known. However, data available from studies conducted among homosexual men indicate that most persons will remain infected.

2. Although asymptomatic, these individuals may transmit HTLV-III to others. Regular medical evaluation and follow-up is advised, especially for individuals who develop signs or symptoms suggestive of AIDS.

3. Refrain from donating blood, plasma, body organs, other tissue, or sperm.

4. There is a risk of infecting others by sexual intercourse, sharing of needles, and possibly, exposure of others to saliva through oral- genital contact or intimate kissing. The efficacy of condoms in preventing infection with HTLV-III is unproven, but the consistent use of them may reduce transmission.

5. Toothbrushes, razors, or other implements that could become contam- inated with blood should not be shared.

6. Women with a seropositive test, or women whose sexual partner is seropositive, are themselves et increased risk of acquiring AIDS. If they become pregnant, their offspring are also at increased risk of acquiring AIDS.

7. After accidents resulting in bleeding, contaminated surfaces should be cleaned with household bleach freshly diluted 1:10 in water.

8. Devices that have punctured the skin, such as hypodermic and acupuncture needles, should be steam sterilized by autoclave before reuse or safely discarded. Whenever possible, disposable needles and equipment should be used.

9. When seeking medical or dental care for intercurrent illness, these persons should inform those responsible for their care of their positive antibody status so that appropriate evaluation can be undertaken and precautions taken to prevent transmission to others.

10. Testing for HTLV-III antibody should be offered to persons who may have been infected as a result of their contact with seropositive individuals (e.g., sexual partners, persons with whom needles have been shared, infants born to seropositive mothers).

Revised recommendations will be published as additional information becomes available and additional experience is gained with this test.

Reported by Centers for Disease Control; Food and Drug Administration; Alcohol, Drug Abuse, and Mental Health Administration; National Institutes of Health; Health Resources and Services Administration

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# ATTACHMENT 15

CDC
CENTERS FOR DISEASE CONTROL AND PREVENTION

November 9, 2001 / Vol. 50 / No. RR-19

MMWR™
MORBIDITY AND MORTALITY WEEKLY REPORT

*Recommendations and Reports*

*Inside:* Two Continuing Education Examinations

# Revised Guidelines for HIV Counseling, Testing, and Referral

# and

# Revised Recommendations for HIV Screening of Pregnant Women

**U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES**
Centers for Disease Control and Prevention (CDC)
Atlanta, GA 30333

# Revised Guidelines for HIV Counseling, Testing, and Referral

***Summary***

*These guidelines replace CDC's 1994 guidelines,* HIV Counseling, Testing, and Referral Standards and Guidelines*, and contain recommendations for public- and private-sector policy makers and service providers of human immunodeficiency virus (HIV) counseling, testing, and referral (CTR). To develop these guidelines, CDC used an evidence-based approach advocated by the U.S. Preventive Services Task Force and public health practice guidelines. The recommendations are based on evidence from all available scientific sources; where evidence is lacking, opinion of "best practices" by specialists in the field has been used.*

*This revision was prompted by scientific and programmatic advances in HIV CTR, as well as advances in prevention and the treatment and care of HIV-infected persons. These advances include a) demonstrated efficacy of HIV prevention counseling models aimed at behavioral risk reduction; b) effective treatments for HIV infection and opportunistic infections; c) effective treatment regimens for preventing perinatal transmission; and d) new test technologies.*

*Although the new guidelines include many aspects of the previous ones (e.g., encouragement of confidential and anonymous voluntary HIV testing, need for informed consent, and provision of HIV prevention counseling that focuses on the client's own risk), the new guidelines differ in several respects, including*

- *giving guidance to all providers of voluntary HIV CTR in the public and private sectors;*
- *using an evidence-based approach to provide specific recommendations for CTR;*
- *underscoring the importance of early knowledge of HIV status and making testing more accessible and available;*
- *acknowledging providers' need for flexibility in implementing the guidelines, given their particular client base, setting HIV prevalence level, and available resources;*
- *recommending that CTR be targeted efficiently through risk screening and other strategies; and*
- *addressing ways to improve the quality and provision of HIV CTR.*

## INTRODUCTION

### Purpose of the Guidelines

These guidelines were developed for policy makers and service providers in the many settings that offer voluntary human immunodeficiency virus (HIV) counseling, testing, and referral (CTR) — public and private, urban and rural, and those with high and low HIV prevalence (Box 1). The guidelines are intended to be used to develop CTR services and policies in traditional clinical settings (e.g., sexually transmitted disease

# ATTACHMENT 16

# Frequency of Indeterminate Western Blot Tests in Healthy Adults at Low Risk for Human Immunodeficiency Virus Infection

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**As part of a phase 1 trial of a candidate AIDS vaccine, blood specimens were collected from 168 healthy adult volunteers at minimal or no risk for becoming infected with human immunodeficiency virus type 1 (HIV-1). These specimens were screened for evidence of HIV-1 infection by enzyme immunoassay (EIA) and the Biotech/Du Pont Western blot ($n$ = 168), culture ($n$ = 122), and polymerase chain reaction assay ($n$ = 20). None of the subjects had a positive test result by any of these assays, but 32% had indeterminate Western blot tests, most of which demonstrated a single band of low intensity. The most common bands were p24 (47%), p55 (34%), and p66 (36%); envelope bands were unusual (gp41, 2%; gp120, 2%). No serum specimen collected after 2–11 months from individuals with indeterminate Western blot results was positive by EIA or Western blot. There was 91% agreement in the test results of the first and second serum samples when the same lot of Western blot kit was used but only 36% agreement when different lots were used. The Biotech/Du Pont Western blot kit thus frequently yields indeterminate test results in the absence of HIV-1 infection, the reproducibility of which is subject to lot-to-lot variability.**

Testing for human immunodeficiency virus type 1 (HIV-1) infection is encouraged for those at high risk and is mandatory for blood and organ donors, for individuals applying for military service, for immigration, and, in many cases, for life and health insurance policies [1]. As routine testing increases, concern has been expressed that the false-positive rate of HIV-1 serologic tests might be unacceptably high among populations with a low prevalence of HIV-1 infection [2]. Two recent studies conducted in low-risk populations concluded that the false-positive rate for detection of HIV-1 infection in the setting of a positive enzyme immunoassay (EIA) and Western blot (WB) was extremely low [3, 4]. However, many studies have noted the occurrence of indeterminate WB tests that have one or more bands but do not meet the criteria necessary for a positive result [4–13]. Indeterminate WB results lead to considerable confusion and anxiety because their meaning is unclear.

As part of a multicentered phase 1 safety and immunogenicity trial of a candidate AIDS vaccine, we screened healthy adults at minimal or no risk for acquiring HIV-1 infection. A surprisingly high percentage, although negative for HIV-1 antibody by commercial EIA, had an indeterminate WB result. Additional WB testing in individuals followed prospectively for 2–11 months allowed us to characterize the pattern of their test results over time and to determine the degree of lot-to-lot variability among different WB kits.

## Materials and Methods

*Participating sites.* Six institutions, designated as AIDS vaccine evaluation units (AVEUs), participated in this study as part of an AIDS vaccine trial. They were located at the Johns Hopkins University School of Hygiene and Public Health (Baltimore) and the Schools of Medicine of Baylor (Houston), Marshall (Huntington, WV), Maryland (Baltimore), Rochester (NY), and Vanderbilt (Nashville, TN) universities. The central laboratory was located at Georgetown University (Washington, DC).

*Subjects.* Healthy volunteers 18–55 years old were screened between December 1987 and April 1988 to determine their eligibility to participate in an AIDS vaccine trial [14]. Volunteers were excluded if they had a history of behavior that placed them at risk for acquiring HIV-1 infection, of hepatitis B virus (HBV) infection or vaccination, or of blood product transfusion within the preceding 6 months. Eligible volunteers were tested by EIA and WB for anti-HIV-1 antibodies ($n$ = 168), by HIV-1 p 24 antigen test ($n$ = 168), and by HIV-1 culture ($n$ = 122). They were also screened for serologic markers of HBV.

Volunteers who had an indeterminate WB result were ineligible to participate in the vaccine trial, regardless of negative tests for

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Informed consent was obtained from all subjects, and experimentation guidelines of the authors' institutions were followed.

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HIV-1 infection by EIA, p24 antigen test, and HIV-1 culture. Thirty-three (62%) of 53 WB-indeterminate volunteers agreed to be retested ~6 months later by EIA, WB, and p24 antigen test for HIV-1 and to be screened for antibodies to human immunodeficiency virus type 2 (HIV-2) and human T-cell lymphotropic virus type I (HTLV-I). Polymerase chain reaction (PCR) for HIV-1 was also done on peripheral blood mononuclear cells (PBMCs) from 20 WB-indeterminate volunteers. During the same period, serial blood samples were collected from study subjects with negative WB results who received placebo or hepatitis B vaccine in the AIDS vaccine trial.

*HIV-1 serologic assays.* Serum samples were tested for HIV-1 antibody and p24 antigen by EIA kits (HIV-EIA and HTLV-III antigen EIA; Abbott Laboratories, North Chicago). WB assays were done with the Biotech/Du Pont kit (Biotech Research Laboratories, Rockville, MD, and Du Pont, Wilmington, DE) and interpreted according to manufacturers' guidelines [6]. Briefly, bands on the WB were assigned an intensity of +/− (less than the intensity of the p24 band on the weakly reactive control strip), + (equal to or greater than the intensity of the p24 band on the weakly reactive control strip but less than that on the strongly reactive control strip), or ++ (equal to or greater than the intensity of the p24 band on the strongly reactive control strip). A WB was defined as positive if it had bands with a + or ++ intensity at p24, p31, and one of the envelope bands gp41, gp120, or gp160. A WB was indeterminate if it had one or more virus-specific bands of any intensity but did not satisfy the criteria for a positive interpretation. The EIA and WB tests were performed at the AVEUs where the respective serum specimens were collected. Paired serum samples were also tested by WB at the central laboratory. To control for observer variability, one investigator at each of the AVEUs and the central laboratory read the WB tests.

*HIV-1 cultures.* Virus was isolated at the AVEUs by a previously described method [15]. Freshly collected PBMCs were separated from heparinized blood and suspended in RPMI 1640 medium containing fetal calf serum, glutamine, penicillin, streptomycin, and interleukin-2. They were routinely cultivated with normal human phytohemagglutinin-stimulated PBMCs. Cultures were assayed for HIV-1 p24 antigen twice a week for ≥4 weeks.

*PCR.* Cryopreserved PBMCs were tested by Dr. Chin-Yih Ou (Centers for Disease Control [CDC]) for the presence of HIV-1 proviral DNA by PCR using the primer pair SK38/39 for the *gag* region of HIV-1, as described previously [16].

*HIV-2 and HTLV-I serologic assays.* Sera were tested by commercially available EIA kits for antibodies to HIV-2 (Genetic Systems, Seattle) and HTLV-I (Du Pont) at Johns Hopkins University.

*Statistical analysis.* Two-tailed Student's $t$ test, $\chi^2$ test, Fisher's exact test, and contingency table analysis were used for statistical analysis. $P \leq .05$ was considered significant.

## Results

None of the 168 healthy adult volunteers at low-risk for HIV-1 infection was positive for HIV-1 antibody by EIA or WB. One volunteer was positive for p24 antigen by EIA, but this result was not confirmed by the HTLV-III antigen neutralization test (Abbott). None of the 122 volunteers screened by HIV-1 culture of their PBMCs had a positive test result, including the individual with the positive p24 antigen test.

Of 168 volunteers tested by WB, 53 (32%) had an indeterminate test result. PBMCs collected on initial or follow-up evaluation from 36 (68%) of these WB-indeterminate individuals were tested for HIV-1 by culture ($n$ = 16), PCR ($n$ = 7), or both ($n$ = 13); all were negative. The groups of individuals with negative ($n$ = 115) and indeterminate WBs ($n$ = 53) were similar in terms of age (mean years ± SD, 34 ± 9 vs. 32 ± 10), sex (male, 56% vs. 49%), race (white, 96% vs. 92%), and serologic markers for HBV (8% vs. 5%). The indeterminate group had the following characteristics: 85% had only bands of low intensity; 68% had a single band (p17, 6%; p24, 28%; p55, 17%; p51, 2%; p66, 15%); 32% had two or more bands (any combination of *gag* bands, 9%; of *pol* bands, 2%; of *gag* and *pol* bands, 17%; of *gag* and *env* bands, 2%; of *gag*, *pol*, and *env* bands, 2%). Bands corresponding to p24, p55, and p66 occurred most frequently (47%, 34%, and 36% of WBs, respectively), and those corresponding to envelope bands occurred least frequently (4%).

Thirty-three (62%) of those with indeterminate WB tests returned for follow-up within 2–11 months (mean, 7 months) (table 1). They did not differ significantly in terms of age, sex, race, or serologic markers for HBV from those with indeterminate WB tests who did not return for follow-up. Their follow-up serum samples were negative by EIA for HIV-1 p24 antigen and antibodies to HIV-1, HIV-2, and HTLV-I. None had developed a positive WB test, including subject 19, who had p24 and gp41 bands detected on initial testing. At the AVEUs, the initial and follow-up serum samples from each individual were tested with different WB kit lots. Only 12 (36%) of 33 had the same indeterminate WB pattern in both serum specimens; 8 had a different indeterminate WB pattern and 13 had a negative WB result.

To determine if this variability in test results was related to the use of different WB kit lots or intrinsic differences in serum samples from the same individual, these serum samples were simultaneously tested at the central laboratory with the same WB kit lot (table 1). Identical results were observed in 30 (91%) of 33 paired serum specimens. The reproducibility of results in paired specimens tested with different kit lots compared with those tested with the same kit lot revealed a marked discrepancy: 12 (36%) compared with 30 (91%) of 33 identical pairs ($P < .001$, $\chi^2$).

The lot-to-lot variability of the WB results was further illustrated in a subset of four subjects who had blood collected on three different occasions over 5–7 months. Each specimen was tested multiple times by different WB kit lots at one of the AVEUs and the central laboratory. In no case was the WB pattern consistently reproduced. The serum specimens of one subject showed WB results ranging from negative to indeterminate with *gag*, *pol*, and *env* bands. This subject's second serum specimen showed the following results: lots 1 and 2, p24; lot 3, negative; lot 4, p24, p31, p51, p66, gp120, gp160. The last result would have met the manufacturer's criteria for positive except that the p24 band was only of +/− intensity.

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**Table 1.** HIV-1 test results from the initial and follow-up evaluation of 33 healthy adults with initially indeterminate Western blot results.

| Subject no., sex | Months between serum collection | Western blot band patterns | | | |
|---|---|---|---|---|---|
| | | AVEUs | | Central laboratory | |
| | | Initial | Follow-up | Initial | Follow-up |
| 1, M | 7 | 17, 24 | 24 | Neg | Neg |
| 2, M | 2 | 24 | 66 | 24, 51, 66 | 24, 55, 66 |
| 3, F | 8 | 24 | 24 | 24 | 24 |
| 4, F | 8 | 17*, 24 | 17 | 17, 24 | 17, 24 |
| 5, F | 7 | 51, 66 | Neg | Neg | Neg |
| 6, F | 8 | 24* | 24* | 24* | 24* |
| 7, M | 7 | 24* | 24* | 24* | 24* |
| 8, F | 6 | 24*, 55 | 24 | Neg | Neg |
| 9, M | 7 | 24, 55 | 55 | Neg | Neg |
| 10, M | 5 | 17, 24, 55 | 24, 55 | 17 | 17 |
| 11, M | 6 | 24 | 24 | 24* | 24* |
| 12, F | 6 | 51, 55, 66 | 51, 66 | 51*, 66* | 51*, 66* |
| 13, F | 5 | 55 | 55 | Neg | Neg |
| 14, F | 6 | 24 | 24 | 24* | 24 |
| 15, M | 6 | 24 | 24 | 24 | 24 |
| 16, F | 5 | 24 | Neg | 24 | Neg |
| 17, M | 7 | 24 | 24 | 24 | 24 |
| 18, F | 5 | 24 | 24, 66 | 24 | 24 |
| 19, F | 8 | 24, 41 | Neg | Neg | Neg |
| 20, F | 11 | 24, 66 | Neg | 24 | 24 |
| 21, M | 11 | 24, 31, 66 | Neg | 24 | Neg |
| 22, F | 7 | 24, 66 | Neg | Neg | Neg |
| 23, M | 7 | 24* | 24* | 24 | 24 |
| 24, F | 9 | 66 | 66 | 66 | 66* |
| 25, M | 6 | 55 | Neg | Neg | Neg |
| 26, M | 9 | 55 | Neg | Neg | Neg |
| 27, F | 9 | 24 | 24 | Neg | Neg |
| 28, F | 6 | 66 | Neg | Neg | Neg |
| 29, M | 9 | 55*, 66* | Neg | Neg | Neg |
| 30, M | 8 | 66 | Neg | Neg | Neg |
| 31, M | 8 | 55 | Neg | Neg | Neg |
| 32, F | 6 | 24 | 24 | 24 | 24 |
| 33, F | 6 | 55 | Neg | 51 | 51 |

NOTE. Initial and follow-up serum samples were negative by EIA for HIV-1, HIV-2, and HTLV-I antibodies and for HIV-1 p24 antigen; specimens from 26 were tested by HIV-1 culture ($n = 8$), polymerase chain reaction ($n = 7$), or both ($n = 11$) and were negative. Initial and follow-up samples were tested sequentially with different WB kit lots at the AIDS vaccine evaluation units (AVEUs) and were subsequently tested simultaneously with the same WB kit lot at the central laboratory. Neg = negative. Numbers refer to proteins.

* Bands of + intensity; all other bands were of +/− intensity.

Serial serum specimens from this subject were repeatedly negative for HIV-1 antibody and p24 antigen by EIA, and PBMCs were negative for HIV-1 by culture and PCR.

The consistency of WB results in subjects WB-negative in initial testing was also examined. Serial serum samples were collected over 7 months from 34 who received placebo or HBV vaccine in the AIDS vaccine trial. The sera were tested by different WB kit lots at both the AVEUs and central laboratory. The WB results for initially WB-negative individuals revealed less internal variance than did those for initially WB-indeterminate subjects. Overall, 467 (98%) of 476 WB tests on 238 serum specimens were negative and 2% were indeterminate. Seven had an indeterminate WB test at some point during the 7-month period. Lot-to-lot variability may also have accounted for these occasional indeterminate WB tests.

## Discussion

Indeterminate WB results have been described in early or advanced HIV-1 infection and in the absence of infection [5–13]. Nevertheless, we were surprised that among the volunteers for an AIDS vaccine trial, one-third of the EIA-negative, healthy adults at low risk for HIV-1 infection had indeterminate WB tests. One individual had a positive p24 antigen test but a negative culture for HIV-1, suggesting that the p24 reactivity may have been nonspecific. All 36 of the WB-indeterminate volunteers tested for HIV-1 infection by culture, PCR, or both were negative. None of 33 WB-indeterminate volunteers who returned for follow-up showed progression to a positive WB, EIA, or p24 antigen test, and none was positive for antibodies to HIV-2 or HTLV-I. These data provide additional support for the CDC recommendation that individuals who have had consistently indeterminate WB tests for at least 6 months should be considered negative for HIV-1 antibodies if they have no known risk factors or clinical symptoms [5].

The indeterminate rate of 32% in our EIA-negative volunteers was higher than the 3%–20% rates previously reported in EIA-negative volunteer blood donors [5, 6, 13]. However, the study that reported the 20% rate would most likely have had a higher rate if it had included WBs with +/− intensity bands in the indeterminate category [13]. Conversely, if we had excluded such WBs from our indeterminate category, only 5% of our volunteers would have had an indeterminate WB test. The frequency of indeterminate WB tests in EIA-positive volunteer blood donors also has varied significantly, from 13% to 48% [4, 7, 10].

Lot-to-lot variability, as described in our study, and the decision to include or exclude bands of faint intensity may contribute to the variation in frequency of indeterminate WB tests. Several studies have noted that follow-up serum specimens from individuals with indeterminate WB tests often have a different band pattern or a negative WB result. In these cases, paired serum samples were tested sequentially and therefore most likely by different WB kit lots [4, 10–13]. One study that demonstrated 100% agreement in band patterns of initial and follow-up serum samples had tested the samples simultaneously with the same WB kit lot [9]. The results of these studies are consistent with our finding of variability among different lots and reproducibility within the same lot of WB kits. One exception is a study that noted poor consistency in WB band patterns of serial serum specimens even when tested simultaneously with the same kit lot [13].

The band composition in our study of EIA-negative, WB-indeterminate subjects was similar to that reported in series of EIA-positive or EIA-negative volunteer blood donors in that most (60%–100%) indeterminate WB tests were com-

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posed exclusively of core bands [4, 10, 13]. The fact that we occasionally noted envelope bands is important in light of the criteria used by the Association of State and Territorial Public Health Laboratory Directors for interpretation of WB tests. These criteria, which have been endorsed by the CDC, specify that a WB test is positive if it has any two of the following bands: p24, gp41, or gp120/160. They do not address band intensity [5]. By these criteria, WB results from three of our volunteers would have been interpreted as positive. None of these low-risk individuals had any clinical or laboratory evidence of HIV-1 infection on initial or follow-up evaluation. It is important to note that these false-positive WB tests were not consistently reproducible, a phenomenon we attributed to the use of different WB kit lots.

Our experience and that of others [7–13] indicates that the overwhelming majority of indeterminate results in low-risk, EIA-negative or EIA-positive individuals are not associated with HIV-1 infection. Our results have implications for both the research and clinical setting. In the area of AIDS vaccine research, we question the need to exclude low-risk volunteers on the basis of an indeterminate WB, especially if they are negative by HIV-1 culture or PCR. In the clinical setting, our findings suggest that the WB, while appropriate as a confirmatory assay for HIV-1 infection in EIA-positive individuals, is not appropriate as a screening test. Furthermore, in light of the considerable lot-to-lot variability, consideration should be given to retesting serum specimens from WB-positive, low-risk individuals by a different WB kit lot to confirm the result.

## Collaborators

Principal collaborators from the NIAID Vaccine Evaluation Units include Stephen Greenberg, Baylor College of Medicine, Houston; Robert Belshe, St. Louis University School of Medicine; Carol Tacket, University of Maryland School of Medicine, Baltimore; Raphael Dolin, University of Rochester School of Medicine and Dentistry, NY; Barney Graham, Vanderbilt University School of Medicine, Nashville, TN; and Donald Stablein, EMMES Corp., Potomac, MD.

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# ATTACHMENT 17

# Risk Factors for Repeatedly Reactive HIV-1 EIA and Indeterminate Western Blots

## *A Population-Based Case-Control Study*

*Connie L. Celum, MD, MPH; Robert W. Coombs, MD, PhD; Margie Jones, MA; Victory Murphy, ARNP; Lloyd Fisher, PhD; Christopher Grant, PhD, DSc; Lawrence Corey, MD; Thomas Inui, ScM, MD; Mark H. Wener, MD; King K. Holmes, MD, PhD*

**Objective:** Causes of indeterminate results of Western blot testing (IWB) for human immunodeficiency virus (HIV) type 1 include seroconversion, HIV-2 cross-reactivity, and autoimmune disease, but most IWB results remain unexplained. This case-control study assessed risk factors for IWB results, including early HIV infection, other retroviral infection, autoantibodies, and other medical conditions.

**Design:** Prospective study to determine HIV seroconversion rate, with a case-control design to assess other risk factors for IWB. Cases (persons with one or more repeatedly reactive HIV-1 enzyme immunoassay with IWB), their current sexual partners, and controls (persons with negative enzyme immunoassay and Western blot results) were recruited from blood banks, health department and prenatal clinics, and private providers in Washington and Oregon.

**Results:** Of 244 cases enrolled, 206 were followed up for 6 months or longer, and six (3.0%; 95% confidence interval [CI], 0.7% to 5.3%) with recent HIV risk behaviors seroconverted. The Western blot banding patterns differed among groups; cases usually had p17 or p24 bands, while controls and cases' sexual partners usually had polymerase bands. Conditional logistic regression indicated that independent risk factors for IWB among male cases and controls were a tetanus booster in the past 2 years (odds ratio, 3.2; 95% CI, 1.2 to 8.6) and sexual contact with a prostitute (odds ratio, 3.0; 95% CI, 1.0 to 9.5). Independent risk factors for women were parity (odds ratio, 1.2; 95% CI, 1.02 to 1.4) and autoantibodies, either rheumatoid factor or antinuclear antibodies (odds ratio, 2.3; 95% CI, 1.03 to 5.6). No cross-reactivity was detected with HIV-2, human T-lymphotrophic virus type 1, feline immunodeficiency or feline leukemia, or bovine immunodeficiency viruses.

**Conclusions:** Evaluation of persons with reactive HIV-1 enzyme immunoassays and IWB should include an assessment of HIV risk and other possible risk factors, such as alloimmunization (ie, parity or recent immunization) or autoantibodies (ie, antinuclear antibodies and rheumatoid factor). The relationship of IWB among men who reported sex with prostitutes is intriguing and warrants further study.

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THE SENSITIVITY and specificity of the human immunodeficiency virus (HIV) type 1 enzyme immunoassay (EIA) are greater than 99%.[1-7] Specimens that are repeatedly reactive by HIV-1 EIA are confirmed by a supplemental test, usually the Western blot, which detects antibodies to denatured HIV-1 proteins. The HIV-1 Western blot has a reported specificity of 98%.[8] Between 4% and 20% of serum samples that are repeatedly reactive by HIV-1 EIA are interpreted as indeterminate by Western blot,[6-9] with the proportion subsequently classified as indeterminate varying according to the immunoblot used, the prevalence of HIV-1 infection in the population tested, and the interpretive criteria used.[8] In reports of EIA-negative volunteers for HIV-1 vaccine trials, up to 33% had indeterminate banding patterns on Western blot.[10,11]

Indeterminate HIV-1 Western blots (IWBs) in HIV-1–infected individuals may result from antibody production against viral core antigens early in HIV-1 infection[12-14] or loss of core antibodies late in HIV-1 infection.[15,16] In HIV-1–negative in-

See Subjects and Methods on next page


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## SUBJECTS AND METHODS

### STUDY POPULATION

This case-control study was initiated at the University of Washington, Seattle, in March 1988.[30] Cases included males and females 16 through 70 years of age with repeatedly reactive EIAs and IWBs in the past, who were referred from testing sites in Washington and Oregon. The HIV-1 Western blot interpretation for Western blots performed on subjects before study enrollment was accepted for study entry. Individuals with a previous diagnosis of HIV seropositivity or acquired immunodeficiency virus (AIDS) and recipients of experimental HIV-1 vaccine were excluded from the study. Cases were recruited by the enclosure of study information with laboratory reports sent to providers with the HIV-1 Western blot result (ie, the IWBs). The rate of response by cases was undefined, given the recruitment from providers and occasional use of anonymous testing. Cases were asked to refer current sexual partner(s) to the study for examination and HIV-1 antibody testing. Controls who had a negative HIV-1 EIA within the past 3 months were recruited from the same HIV-1 testing sites and were matched by HIV-1 testing site.

Because some cases referred with IWBs had negative Western blot tests at the time of study entry, and some persons selected for controls based on a nonreactive EIA had IWB, a subanalysis was done in which such cases and controls were excluded to compare cases with repeatedly reactive HIV-1 EIA and IWBs with controls who were negative for HIV-1 by both EIA and Western blot. We thus performed a subset analysis of cases with repeatedly reactive EIAs and IWBs and controls with nonreactive EIA and negative Western blot results at visit 1, the time at which information on independent variables was also collected.

After informed consent was obtained at the first study visit, cases and controls were interviewed about history of sexually transmitted diseases, number of sexual partners in the past 3 months and 1 year, HIV risk behaviors since 1978, and general medical history, and they were examined. Medical history included questions about parity, autoimmune illness (defined as thyroiditis, juvenile-onset diabetes, lupus erythematosus, and rheumatoid arthritis), liver disease, immunizations, recent viral-like illness, and exposure to cats and farm animals.

### STUDY DESIGN

Cases who did not seroconvert within 6 to 9 months of follow-up were compared with EIA-negative controls to ascertain possible risk factors for IWB other than acute HIV-1 seroconversion. The Centers for Disease Control and Prevention recommends 6 months of serologic follow-up for persons with IWBs.[8] In an analysis by Horsburgh et al,[31] 95% of persons seroconverted within 6 months after sexual exposure to HIV-1.

### LABORATORY TESTING

The University of Washington Virology Laboratory and the Washington State Public Health Laboratory, Seattle, performed HIV-1 EIAs and Western blots for the study. Both laboratories subscribe to the College of American Pathologists Proficiency Panel for HIV-1 antibody testing. Cases were followed up prospectively, with HIV-1 EIAs and Western blots repeated every 3 months for 6 to 9 months to detect seroconversion. Controls were tested once at the time of study enrollment for HIV-1 EIA and Western blot.

Enzyme immunoassays (Dupont, Biotech Research Laboratory Inc, Rockville, Md; and Genetic Systems, Seattle, Wash) and Western blot testing (Epitope, Beaverton, Ore) were performed on study subjects. The Centers for Disease Control and Prevention interpretive criteria were used for Epitope Western blots; a Western blot was considered positive if antibodies were present to *two* of the following HIV-1 viral proteins: p24, gp41, and gp120 or gp160.[8]

dividuals, IWBs have been shown to result from cross-reactive antibody to HIV-2[17-21] or cross-reactive autoantibodies and alloantibodies.[22-25] Etiologies of IWBs have not been well characterized and have been assessed primarily in case series and case reports, which have reported associations of IWBs with medical problems, including T-cell lymphoma, multiple sclerosis, and dermatologic disorders,[22] injection drug use,[26] alcoholic liver disease,[27] and autoimmune diseases, such as Sjögren's disease.[28]

Individuals with a reactive HIV-1 EIA and IWB are currently excluded from donating blood and have had difficulty obtaining life and disability insurance, US immigration status, and visas for foreign travel, regardless of their risk history. Persons notified of a reactive HIV-1 EIA and IWB are often concerned not only about their risk of HIV seroconversion but also about whether the IWB reflects any underlying medical condition.[29] This population-based case-control study assessed risk factors for IWB; persons with repeatedly reactive EIAs and IWBs with and without risk factors for HIV infection were studied. We found that IWBs were associated with a low risk of HIV seroconversion and that the major risk factors identified for IWBs related to alloimmunization or autoantibodies.

## RESULTS

Two hundred forty-four persons with IWBs for HIV-1 were referred and enrolled in the study as of August 1991. Forty-


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Western blots without any bands were interpreted as negative, and blots with bands not meeting the criteria for a positive blot were interpreted as indeterminate. The presence of nonviral bands only was not considered an IWB for the case-control analyses.[32] The diagnosis of HIV-1 infection was based on seroconversion to a positive Western blot with persistently reactive EIA. Positive Western blots were repeated. An HIV-1 culture, polymerase chain reaction, serum p24 antigen, and a recombinant p24, gp41 EIA (Syva Microtrak, Syva Corp, Palo Alto, Calif) were performed on study subjects with positive Western blots, as previously described.[30]

The screen for autoantibodies included antinuclear antibodies (ANAs) and rheumatoid factor. Testing for ANAs was performed by means of an initial 1:40 serum dilution on rat liver and 1:100 dilution on HEp-2 cells. Latex agglutination was used to test for the presence of rheumatoid factor. A titer of 1:40 or more was considered a positive ANA or rheumatoid factor. Lymphocyte subset analyses were performed by flow cytometry. The screen for other infectious diseases included a serum VDRL, antibody to herpes simplex types 1 and 2 by Western blot,[33] and hepatitis B surface antigen and antibody. The EIAs for human T-lymphotrophic virus (HTLV) type 1 and HIV-2 were assessed in 116 and 91 cases, respectively. To ascertain possible cross-reactivity with animal retroviruses, serum from the subset of the first 27 cases who reported raw milk ingestion or farm animal contact were tested for antibodies to bovine leukemia and bovine immunodeficiency virus by p24 agar immunodiffusion and Western blot. Serum samples from the first 26 cases who owned a pet cat were tested for reactivity on Western blots for feline leukemia virus and for feline immunodeficiency virus.[34]

Serum samples from 78 cases were screened for the presence of antibodies to class I HLA antigens by means of a panel of T lymphocytes from 50 donors of known HLA type. Serum samples were screened for the presence of antibodies to class II HLA antigens by means of a panel of B lymphocytes from 25 donors of known HLA type. A modified microlymphocytotoxicity assay was used to detect complement-fixing antibody.[35]

## STATISTICAL METHODS

Demographic and HIV risk factors were compared by the $\chi^2$ test and Fisher's Exact Test for categorical data and Student's *t* test for continuous data. The Mann-Whitney test was used for comparing continuous distributions when the assumption of a normal distribution was not appropriate. Ninety-five percent exact binomial confidence intervals (CIs) for the seroconversion risk were calculated.

To assess risk factors for IWBs other than HIV-1 infection, the nonseroconverter cases and controls were compared in terms of medical history and risk behaviors for HIV since 1978. Conditional logistic regression was used to compare the nonseroconverter cases and controls for risk factors for IWBs, stratified by testing site. Cases without well-matched controls (ie, cases referred from private providers, immigration, or life and disability insurance screening) were not included in the logistic regression, which was conditioned on testing site. Variables with $P<.1$ on univariate analysis were considered for possible entry in forward stepwise multivariate runs with the use of conditional logistic regression. Analyses were also performed for males and females combined and separately.

A second set of conditional logistic regression analyses were performed in which only the cases who were still repeatedly reactive on EIA and had IWBs at visit 1 and controls who were both EIA and Western blot negative at visit 1 were included to reduce potential misclassification bias from inclusion of EIA- or Western blot–negative cases and EIA-negative and Western blot–indeterminate controls. The female cases and controls referred from prenatal clinics were also excluded from the second analyses to avoid the potential confounder of current pregnancy in examining the relationship of parity to IWB.

A third logistic regression analysis was performed among blood donor cases and controls only, excluding cases and controls from all other testing sites.

seven cases were referred from private physicians, life and disability insurance screening, and military screening and were excluded from the case-control comparisons because of the inability to recruit controls randomly from the same testing sites. Of the remaining 197 cases, 76 (39%) were from health department clinics, including the Sexually Transmitted Diseases Clinic and AIDS Prevention Project, 106 (54%) were referred from blood banks in Washington and Oregon, and 15 (8%) were referred from prenatal and women's clinics.

The risk of seroconversion was determined for cases with more than 6 months of follow-up, as described above. Of the 244 cases, 206 (84%) were followed up with repeat HIV-1 EIA and Western blots at least 6 months apart. The remaining 38 cases were followed up for less than 6 months because of unavailability for follow-up (n=26), incarceration (n=3), and residence out of the area (n=9). The cases with less than 6 months of follow-up were significantly more likely to be African-American, never have been married, be injection drug users, and have an income less than $20 000 than were the cases with 6 months or longer follow-up (data not shown). The risk of seroconversion was six (3.0%) of 206 (95% CI, 0.7% to 5.3%), and all six individuals had recent HIV risk behavior. We previously reported on 89 of these individuals, of whom four (4.5%) seroconverted.[30] The risk of seroconversion may be underestimated by the unavailability for follow-up of 11 injection drug users.

One hundred thirty-one EIA-negative controls were recruited from the same HIV-1 testing sites as the cases:


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77 controls (59%) were recruited from blood banks, 48 (37%) from the AIDS Prevention Project and Sexually Transmitted Diseases Clinic, and six (5%) from prenatal and women's clinics.

Eighty-three current sexual partners of 83 cases were enrolled in the study. Forty (48%) were partners of index cases who were blood donors, 18 (22%) had partners from the AIDS Prevention Project or the Sexually Transmitted Diseases Clinic, four (5%) were male partners of women referred from prenatal clinics, 12 (14%) were from private providers, eight (10%) were from insurance screening, and one (1%) was from immigration screening.

## HIV-1 SEROLOGIC FINDINGS AMONG CASES, CONTROLS, AND CASES' SEXUAL PARTNERS

Results of HIV-1 EIA and Western blots from the first study visit among the 244 cases, 131 controls, and 83 cases' sexual partners are shown in **Table 1**. Of 244 cases referred to the study because of previous reactive EIA and IWB, 139 still had repeatedly reactive EIA at study visit 1, and of these, 124 also had IWBs at the first study visit and did not seroconvert to a positive Western blot. Five of the six seroconverters had a positive Western blot at visit 1, which was 1 month or less after they first tested indeterminate with a p24 band only initially. The seroconverters were confirmed to be HIV positive by supplemental tests, including HIV-1 culture, polymerase chain reaction, serum p24 antigen, and recombinant p24, gp41 EIAs.[30] Two low-risk cases and one case with multiple heterosexual partners had false-positive Western blots during follow-up, with the presence of p24 and envelope bands, but were determined not to be HIV infected through HIV-1 culture, polymerase chain reaction, and recombinant envelope EIAs. Of the 131 EIA-negative controls, 35 (27%) had IWBs and six were recruited from prenatal and women's clinics. The remaining 90 controls with negative immunoblots were included as controls for the second set of risk factor analyses, as described above. Banding patterns included p17 and p24 (core) bands in 138 (88%) of the 156 cases who had IWBs at study visit 1 compared with 19 (54%) of the 35 controls with IWBs and three (14%) of the 21 cases' sexual partners with IWBs ($P<.001$). Five partners of the cases had repeatedly reactive EIAs; one was negative by Western blot and one was indeterminate with a p55 band. Three (4%) of the cases' sexual partners were confirmed HIV-seropositive by Western blot, two of whom had HIV risk behaviors (a bisexual man and a hemophiliac). The third partner was married to a recent immigrant from West Africa who had a stable p17 band on Western blot.

## RISK FACTORS FOR IWB AMONG THE CASES

We first analyzed potential causes of IWBs for HIV-1 among the nonseroconverter cases only. These factors included cross-reactivity with HIV-2, HTLV-1, feline and bovine retroviruses, HLA antibody cross-reactivity, and current pregnancy. We then compared cases and controls for potential risk factors for IWB on the basis of historical data (eg, autoimmune history, parity, and high-risk behavior), as well as laboratory data (eg, autoantibodies, hepatitis B and herpes simplex type 2 antibodies, and lymphocyte subsets).

**Table 1. Results of HIV-1 EIA and Western Blots in Cases, Controls, and Cases' Sexual Partners at First Study Visit***

| | Cases (n=244) | Controls (n=131) | Cases' Sexual Partners (n=83) |
|---|---|---|---|
| EIA† repeatedly reactive, No. (%) | 139 (57) | 0 (0) | 5 (6) |
| Epitope Western blot,‡ No. (%) | | | |
| Negative | 83 (34) | 96 (73) | 59 (71) |
| Indeterminate | 156 (64) | 35 (27) | 21 (25) |
| p17 only | 61 | 5 | 2 |
| p24 only | 67 | 13 | 1 |
| p17 and p24 bands | 10 | 1 | 0 |
| env only | 2 | 1 | 1 |
| Other bands | 9 | 7 | 6 |
| Non-HIV bands | 7 | 8 | 11 |
| Positive‡ | 5 (2) | 0 (0) | 3 (4) |

*HIV indicates human immunodeficiency virus; EIA, enzyme immunoassay.

†Genetic Systems (Seattle, Wash) or Dupont (Biotech Research Laboratory Inc, Rockville, Md) EIAs and Epitope (Beaverton, Ore) Western blots were performed for the study subjects on serum samples obtained at the first study visit. Western blots were interpreted as positive by Centers for Disease Control and Prevention interpretive criteria (if at least two of the following anti-HIV antibodies were present: p24, gp41, and/or gp120/160).

‡Five of the six seroconverters had a positive Western blot at the first study visit within 1 month after the initial indeterminate Western blot; all six seroconverters had been referred with a p24 band on initial blot.

## CROSS-REACTIVITY AMONG OTHER HUMAN AND ANIMAL RETROVIRUSES

Of 116 cases tested, only one had a weakly reactive HTLV-1 EIA and an IWB for HTLV-1 with p19 and p29 bands. Ninety-one cases tested for HIV-2 by a synthetic peptide for HIV-2 gp36 were negative. Two cases with residence in West Africa or West African sexual partners were tested for HIV-2, one by HIV-2 EIA and radioimmunoprecipitation assay and one by HIV-2 polymerase chain reaction; both were negative.

Among 27 cases who reported raw milk ingestion or farm animal contact, none had antibodies to bovine immunodeficiency virus. All of the first 26 cases who reported a cat as a pet were tested by Western blot for antibodies to feline leukemia virus and to feline immunodeficiency virus. Eight (31%) of 26 had bands on Western blot corresponding to p15e of feline leukemia virus, the transmembrane protein, compared with four (29%) of


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14 controls (*P*=.8). No cases or controls had antibodies against feline immunodeficiency virus.

## HLA ANTIBODIES

In 78 cases HLA antibodies were tested; anti–class I HLA reactivity was found in 13 (17%), and anti–class II HLA reactivity was not identified in any of the samples. All but one of the cases who demonstrated anti–class I HLA reactivity were multiparous women. There was no correlation between HLA antibodies, a positive ANA or rheumatoid factor, and presence of p17 or p24 banding patterns on Western blot.

## IWB AMONG PREGNANT WOMEN

Of the total sample of 125 female cases referred to the study, 15 (13%) were pregnant when screened for HIV antibody and when enrolled into the study. Seven of the 15 pregnant patients had risk factors for HIV, but none seroconverted. Of the seven women with postpartum serum samples available for HIV serologic testing, six had persistent indeterminate banding patterns on Western blot a mean (±SD) of 5.3±3.6 months post partum. The one woman whose Western blot turned negative after delivery had a positive rheumatoid factor (titer, 1:160) that also became negative post partum.

## RISK FACTORS FOR IWB: CASE-CONTROL COMPARISONS

Cases and controls were compared in terms of demographics and HIV risk factors, medical history (ie, autoimmune history, parity, immunizations), and laboratory tests (ie, ANAs, rheumatoid factor, hepatitis B and herpes simplex type 2 serologic tests, and lymphocyte subsets). The demographics and HIV risk factors of the 191 cases and the 131 controls, matched by testing site, were similar except that the cases had less education and were less likely to have had a previous sexually transmitted disease (**Table 2**).

The medical histories and results of laboratory studies of the 191 cases and 131 controls were similar except that the cases were more likely to have received a tetanus booster in the past 2 years (22% vs 9%; *P*=.005), have had a positive tuberculin skin test (9% vs 3%; *P*=.04), and have a positive rheumatoid factor (6% vs 0.8%; *P*=.05). There was a trend toward a higher prevalence of positive ANAs among the cases (27%) than the controls (14%; *P*=.14). The cases with autoantibodies in our study usually had low-titer ANAs (median titer, 40; range, 40 to 640) with a speckled or homogeneous pattern and moderate titers of rheumatoid factor (median titer, 320; range, 40 to 10 240). The absolute CD4 count did not differ between cases and controls (median, 1.020 and $1.126\times10^9$/L [1020 and 1126/μL], respectively, for cases and controls). The prevalence of hepatitis B surface antibody was similar between cases and controls (11% and 13%, respectively). The prevalence of antibody to herpes simplex virus type 1 was 26 (72%) of 36 cases tested and 13 (56%) of 23 controls tested. The prevalence of herpes simplex virus type 2 specific antibody was eight (22%) of the 36 cases and four (17%) of the 23 controls (*P*=.61 for differences in herpes simplex virus type 2 seropositivity).

Multivariate analyses were performed separately for females and males to examine gender-specific risk factors for IWB (**Table 3**). The female cases were more likely to be parous (odds ratio [OR], 1.2; 95% CI, 1.02 to 1.4) and to have autoantibodies (either ANAs or rheumatoid factor; OR, 2.3; 95% CI, 1.03 to 5.6). The male cases were more likely to have received a tetanus booster in the past

**Table 2. Demographic Characteristics and HIV Risk Factors of Cases and Controls***

| | Cases† (n=191) | Controls (n=131) | *P* |
|---|---|---|---|
| Age, y, median (range) | 37 (13-73) | 35 (17-69) | .50 |
| Sex, No. (%) F | 104 (54) | 63 (48) | .31 |
| Race, No. (%) W | 168 (88) | 112 (86) | .63 |
| Marital status, No. (%) | | | |
| Never married | 63 (33) | 52 (40) | .43 |
| Married | 90 (47) | 56 (43) | |
| Divorced/widowed | 38 (20) | 23 (18) | |
| Education, y, median (range) | 14 (0-23) | 15 (5-25) | .01 |
| Annual family income ≥$20 000, No. (%) | 108 (57) | 64 (49) | .15 |
| Sexual preference, No. (%) | | | |
| Heterosexual | 173 (91) | 107 (82) | .48 |
| Bisexual | 8 (4) | 7 (5) | |
| Homosexual | 6 (3) | 12 (9) | |
| Never sexually active | 4 (2) | 5 (4) | |
| No. of sexual partners, median (range) | | | |
| Past 3 mo | 1 (0-20) | 1 (0-6) | .37 |
| Past year | 1 (0-100) | 1 (0-40) | .38 |
| Past STDs, ‡ No. (%) | 66 (35) | 59 (45) | .09 |
| High-risk sexual partner since 1978, No. (%) | 49 (26) | 36 (27) | .81 |
| Sexual contact with prostitute since 1978, No. (%) | 16 (8) | 6 (5) | .27 |
| Past prostitution, No. (%) | 8 (4) | 3 (2) | .55 |
| Transfusion between 1978-1985, No. (%) | 9 (5) | 4 (3) | .65 |
| Past injection drug use, No. (%) | 16 (8) | 8 (6) | .58 |
| Any risk for HIV since 1978, No. (%) | 56 (29) | 41 (31) | .79 |

**HIV indicates human immunodeficiency virus; STD, sexually transmitted disease.*

*†Cases in this table are restricted to the nonseroconverting cases for whom controls were available for matching by testing site (eg, blood centers, high-risk testing sites, ie, Seattle–King County AIDS Prevention Project and Sexually Transmitted Diseases Clinic), and prenatal clinics. The six cases who seroconverted and the 47 cases referred from private providers, life and disability insurance screening, and military screening, for whom site-matched controls were not available, were excluded from the table.*

*‡Including a self-reported history of gonorrhea,* Chlamydia, *herpes, and syphilis.*


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**Table 3. Analyses of Risk Factors for Indeterminate HIV-1 Western Blots***

| | Cases | Controls | Univariate OR (95% CI) | Multivariate OR (95% CI) | P |
|---|---|---|---|---|---|
| | | Females | | | |
| N | 104 | 63 | ... | ... | ... |
| Parity, No. (%) | 80 (77) | 37 (59) | 1.2 (1.0-1.4) | 1.2 (1.02-1.4) | .03 |
| Autoantibodies (ANA or rheumatoid factor), No. (%) | 33 (32) | 11 (17) | 2.3 (1.1-5.0) | 2.3 (1.03-5.6) | .04 |
| | | Males | | | |
| N | 87 | 68 | ... | ... | ... |
| Tetanus booster, past 2 y | 21 (24) | 6 (9) | 3.1 (1.2-8.2) | 3.2 (1.2-8.6) | .02 |
| Sexual contact with prostitute since 1978, No. (%) | 15 (17) | 5 (7) | 2.8 (0.9-8.9) | 3.0 (1.0-9.5) | .06 |

*Comparisons of 191 nonseroconverter cases and 131 controls. Cases in this table are restricted to the nonseroconverting cases for whom controls were available for matching by testing site (eg, blood centers, high-risk testing sites, ie, Seattle–King County AIDS Prevention Project and Sexually Transmitted Diseases Clinic), and prenatal clinics. The six cases who seroconverted and the 47 cases referred from private providers, life and disability insurance screening, and military screening, for whom site-matched controls were not available, were excluded from the table. HIV-1 indicates human immunodeficiency virus type 1; OR, odds ratio; CI, confidence interval; and ANA, antinuclear antibodies.

2 years (OR, 3.2; 95% CI, 1.2 to 8.6) and to have had sexual contact with a prostitute since 1978 (OR, 3.0; 95% CI, 1.0 to 9.5).

To reduce potential bias from misclassification, we performed a second set of multivariate analyses excluding cases with a negative HIV-1 EIA or Western blot at the time of study enrollment and controls with IWBs at visit 1 (**Table 4**). We also excluded cases referred from prenatal clinics, because current pregnancy could serve as a confounder in looking at parity as a risk factor for IWB. This final subset included 74 cases and 90 controls. The demographics of the 74 nonseroconverter cases who were EIA reactive and had IWBs at visit 1 and the 90 EIA- and Western blot–negative controls were similar, except that the median number of years of education was lower for cases than controls, and a higher proportion of cases reported sexual contact with a prostitute since 1978 (data not shown).

Gender-specific analyses of these 74 cases and 90 controls showed results similar to those obtained in Table 3, except that CIs were wider with the smaller sample. Risk factors implicated for IWBs among females were parity (OR, 1.3; 95% CI, 1.1 to 1.7) and autoantibodies (OR, 2.4; 95% CI, 0.8 to 6.6); among males the risk factor was sex with a prostitute (OR, 3.8; 95% CI, 0.9 to 16.4).

Analyses limited to blood donor cases and controls found that significant risk factors were tetanus booster in the past 2 years (OR, 2.3; 95% CI, 0.9 to 5.8) and autoantibodies (OR, 2.0; 95% CI, 0.98 to 4.0). Among female blood donor cases and controls, cases were more likely to be parous (77% vs 47%; OR, 3.0; 95% CI, 1.1 to 8.2).

## COMMENT

With increasing pressure to screen more of the population for antibodies to HIV-1,[36] more indeterminate HIV-1 serologic results will be generated. Previous studies that have examined causes of IWBs other than acute HIV-1 seroconversion or HIV-2 infection consisted of case series primarily of low-risk persons, such as blood donors or persons with known autoimmune illnesses.[22-25,28,37] This is the first case-control study to examine risk factors for IWBs for HIV-1 in persons at either high or low risk for HIV infection. We compared both high- and low-risk cases with controls for medical conditions and exposures that might result in autoantibodies or alloimmunization, and prospectively followed up the cases for 6 months or longer to determine whether they seroconverted to a positive Western blot. The risk of seroconversion was 3.0%, comparable with that of earlier reports in blood donor cohorts,[23-25,37] and seroconversion was observed only among persons with recent high-risk behavior.

Immunoblot banding patterns of cases differed from those of the controls and the current sexual partners of the cases. Cases more often had anti-core (p17 or p24) antibodies. In previous studies, up to 33% of EIA-nonreactive low-risk individuals had IWBs, often with p24 or polymerase reactivity.[11,12] Thus, it appears that EIA screening followed by Western blot testing of EIA-reactive serum samples tends to select IWB patterns with antibody to core epitopes. Although core epitopes tend to be retrovirus-group specific, we found no association of HIV-1 IWBs with serologic reactivity to other known human and animal retroviruses (ie, HTLV-1, HIV-2, or bovine or feline leukemia or immunodeficiency viruses). Similarly, a recent study of 99 Minnesota blood donors with IWBs for HIV-1 also found no evidence of HIV-1 or HIV-2 infection.[37] One case series from the New York Blood Center in Syracuse suggested an association between indeterminate HIV-1 immunoblots and bovine immunodeficiency virus, but this was not subsequently confirmed.[24]


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**Table 4. Subset Analyses of Risk Factors for Indeterminate HIV-1 Western Blots***

| | Cases (n=74) | Controls (n=98) | Univariate OR (95% CI) | Multivariate OR (95% CI) | P |
|---|---|---|---|---|---|
| | | Females | | | |
| N | 43 | 44 | ... | ... | ... |
| Parity, No. (%) | 31 (72) | 22 (51) | 1.3 (1.1-1.7) | 1.3 (1.1-1.7) | .02 |
| Autoantibodies (ANA and rheumatoid factor) | 15 (36) | 8 (20) | 2.4 (0.8-6.6) | ... | ... |
| | | Males | | | |
| N | 31 | 46 | ... | ... | ... |
| Sex with a prostitute | 8 (26) | 3 (7) | 3.8 (0.9-16.4) | 3.8 (0.9-16.4) | .07 |

*Comparisons restricted to nonseroconverter cases (enzyme immunoassay [EIA]) repeatedly reactive and indeterminate Western blot (IWB) and EIA- and Western blot–negative controls at visit 1. To reduce potential misclassification from inclusion of cases who were EIA and/or Western blot negative and controls who were EIA nonreactive but had IWB, the risk factors for IWB were compared between the nonseroconverter cases who were EIA repeatedly reactive and IWB and the EIA- and Western blot–negative controls, using serologic results from visit 1. Cases and controls referred from prenatal clinics were also excluded (see text). HIV-1 indicates human immunodeficiency virus type 1; OR, odds ratio; CI, confidence interval; and ANA, antinuclear antibodies.

Risk factors for IWB among the nonseroconverter female cases in the present study were parity and autoantibodies, suggesting cross-reactive antibody. The association with parity persisted even after cases and controls from prenatal clinics were excluded. Parity could reflect alloimmunization during pregnancy, producing antibody to cellular proteins that comigrate with HIV proteins on the Western blot. False-positive HIV EIA tests in multiparous women have been associated with the presence of HLA-DR4 antibodies.[38,39] Although 12 of 13 cases in our study with anti–class I HLA reactivity were multiparous women, we found no significant relationship of HLA antibodies to IWBs, and anti–class II reactivity was not observed. Golding and colleagues[40] demonstrated DNA homology between HIV-1 gp41 and human class $\beta_1$ domains; however, most of our cases had cross-reactivity to core proteins, either p17 or p24. We found no independent association between pregnancy at the time of HIV testing and IWBs, but our power to detect an association was only 37% given the sample size of female cases and controls. Since HIV testing of pregnant women is increasing, and if pregnancy is a risk factor for IWB, rapid evaluation protocols and counseling will be necessary to avoid unnecessary anxiety related to an indeterminate test result and to determine the HIV status of the mother and fetus.[32]

Autoantibodies have been detected in approximately 20% of the population[41] and were found in 37% of our cases and 21% of our controls. Autoantibodies might cross-react with normal cellular determinants of the H-9 or CEM cells in which HIV-1 is cultured before Western blot production. Of 22 cases with a reported autoimmune history, 13 (59%) reported a history of autoimmune thyroid disease. However, we did not specifically measure antimicrosomal antibodies or antithyroglobulins. There was no correlation between a reported history of autoimmune disease and the presence of autoantibodies tested (ANA or rheumatoid factor). Although a link between human retroviruses and certain autoimmune illnesses, such as Sjögren's disease and systemic lupus erythematosus, has been suggested, only limited data support this hypothesis.[28,42-46]

Among men, a recent tetanus booster and sex with prostitutes were implicated as risk factors for IWBs. A recent tetanus booster could result in polyclonal stimulation of B cells and production of antibody with cross-reactivity to epitopes of HIV-1 or cellular proteins. An association of trivalent influenza vaccine with false-positive EIAs and IWBs has been reported among blood donors[47,48]; we sought but did not observe an association with recent influenza vaccination in our study (data not shown).

Because sexual contact with a prostitute was a risk factor for IWBs among men, we evaluated hepatitis B surface antibody, herpes simplex virus type 2 seropositivity, and history of sexually transmitted diseases as surrogate markers for sexual activity and sexually transmitted disease risk.[49-51] All of these were similar among cases and controls. Furthermore, the percentage of IWBs among sexual partners of cases was not higher than that among controls, also arguing against a sexually transmissible infection. Further studies would be needed to investigate whether a sexually transmitted infection accounts for the association between prostitute contact and IWBs among the male cases. Sex with a prostitute was not a significant risk factor for IWBs among blood donor cases. This result corroborates the finding by Dock and colleagues[25] in which blood donors with IWBs and HIV-1–seropositive and HIV-1–seronegative blood donor controls had similar rates of serologic evidence of past cytomegalovirus, Epstein-Barr virus, hepatitis A and B virus, or herpes simplex virus 1 infections.

There are several limitations to our study. We recruited a population-based sample of high- and low-risk individuals with IWBs referred from the community, and we recruited EIA-negative controls from the same testing sites to match for the heterogeneous mix of high- and


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low-risk cases. Since we were not able to recruit EIA-negative controls from private providers and insurance companies, we did not include those cases in the case-control comparisons of risk factors for IWB. Case-control studies involve examination of exposures and risk factors for a relatively rare disease outcome. The use of a case-control method to examine risk factors for a reactive laboratory test that is not always reproducible over time or with different manufacturers' tests presented methodologic issues. To reduce potential misclassification from inclusion of cases who no longer had IWBs at the first study visit and controls who had IWBs, we performed subset analyses including only those cases who still had repeatedly reactive EIAs and IWBs at visit 1 and the controls who were both EIA nonreactive and Western blot negative. The ORs for risk factors identified in the initial and subset analyses were similar, but power was sacrificed in the subset analysis.

Our data have four principal implications for facilitating the management of IWBs for HIV in the clinical setting. First, approximately one third of persons who present with IWBs will not be repeatedly reactive by follow-up EIA, and retesting within 1 month to identify this group is warranted.[30] There is no need for further testing or follow-up of this group. Second, 3% of persons with IWBs in this study were infected with HIV-1, and the most important factor for seroconversion was high-risk behavior, which could be determined from the risk history. As previously reported,[30] the Western blot banding pattern and selective supplemental testing for HIV can be used to counsel patients and identify those at greatest risk of seroconversion. Supplemental testing and repeat Western blot testing were also useful in identifying persons with no history of high-risk behaviors who proved to have false-positive Western blots in this study. Third, no cross-reactivity with known human retroviruses or animal retroviruses was demonstrated in our study population. However, as additional retroviruses are identified, serologic and virologic studies will be of interest among persons with reactive HIV-1 EIAs and indeterminate HIV-1 serologic findings, particularly since the antibody pattern observed most often in cases with IWBs suggested antibody to core proteins.

Fourth, the significant risk factors for IWBs identified in our study included parity and autoantibodies among the female cases, and tetanus booster in the past 2 years and sexual contact with a prostitute since 1978 among the male cases. Medical history will identify subjects with such risk factors. Study of persons with IWBs should include an assessment of HIV risk behavior as well as these potential risk factors by history and testing for autoantibodies.

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# ATTACHMENT 18

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# MINIREVIEW

# Frequency, Causes, and New Challenges of Indeterminate Results in Western Blot Confirmatory Testing for Antibodies to Human Immunodeficiency Virus▽

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More than 2 decades of developments in the field have led to a wide variety of test kits available for addressing the different needs of surveillance, diagnosis, and monitoring of human immunodeficiency virus (HIV) infection and antiretroviral therapy (20, 23). These test kits are based on different technologies, including immunoassays and molecular testing. Typically, enzyme-linked immunosorbent assays (ELISAs) and rapid tests for detecting anti-HIV antibodies are used for blood screening and/or surveillance purposes. The former include third-generation ELISAs that utilize the antigen sandwich design for improved sensitivity, while the latter term refers to immunochromatographic tests or other simple antibody tests requiring less than 30 min of assay time. Since the viral capsid (core) p24 protein can be detected earlier than anti-HIV antibodies (93), attempts had also been made to develop a new generation of ELISA capable of simultaneously detecting the p24 antigen and anti-HIV antibodies (34, 52, 76, 101, 103). Significant reductions of the window period were achieved, which is ideal for blood screening and surveillance (102), but users were also advised to be mindful of possible limitations when utilizing such a combination assay (52, 81, 83). With specific panels, a delay in detection by a fourth-generation ELISA was evident compared with a dedicated p24 antigen assay (81). This shortcoming of fourth-generation ELISAs is understandable, because the surface area of the solid phase for detection is constant and limited regardless of whether it is utilized for a single test or combined assays (101). It should also be noted that an additional procedure for the dissociation of immune complexes employed by dedicated p24 antigen assays but not by combined assays was found to facilitate sensitive detection (85). However, this does not mean that using ELISA dedicated for detecting p24 antigen has no shortcomings. The viral p24 protein is a transient marker that can fall to an undetectable level after initial acute infection and only reemerges at the advanced stage of AIDS in patients (93, 107). Hence, tests utilizing this marker are more valuable for situations such as screening blood products from individuals before antibodies are produced, detecting early infections in persons who have been exposed but are seronegative, or monitoring antiviral therapy (20). In this respect, the diagnostic value of a p24 antigen assay is restricted to detecting infections in newborns of HIV-positive mothers or individuals who have had very recent high-risk exposure (59). In fact, dedicated p24 antigen tests add very little to safety when used in blood screening (1), especially in view of the advances made with molecular testing (22). For even earlier detection, nucleic acid tests were often found to have a clear advantage in sensitivity over p24 antigen assays (22, 51, 84) or other immunoassays (66). The nucleic acid test approach utilizes molecular technologies, such as in situ hybridization, reverse transcription-PCR, nucleic acid sequence-based amplification, and branched-chain DNA, for the detection of viral nucleic acid, such as viral RNA or proviral DNA. In particular, by measuring the levels of viral RNA, the number of HIV virions in the blood (viral load) can be established. Therefore, RNA testing is a valuable tool, not only for the early detection of infections, but also (more commonly) for monitoring disease progression and antiretroviral therapy. However, one must be mindful of the fact that the approach using RNA testing has not been readily accepted by the relevant authorities for the purpose of diagnostics (17, 20) until very recently. To date, only one RNA qualitative assay has been approved by the U.S. FDA for used as an aid in the diagnosis of HIV type 1 (HIV-1) infection, including primary or acute infection (http://www.fda.gov/cber/approvltr/hivhcvgen100406L.htm). The precaution and exclusion are primarily due to the uncertainty in providing a correct diagnosis under certain circumstances. The shortcomings of HIV RNA testing include variability in general in viral-load measurements, inconsistency in performance when testing different genotypes or clades, and sensitivity to inhibiting substances sometimes present in specimens that may result in false negatives (60, 86). In addition, there were reported cases of false positives (FPs), suggesting that HIV viral-load testing is an inappropriate tool for the diagnosis of acute HIV infection (70). Furthermore, the lack of a universally recognized standard for quantification impedes a valid comparison of results from different assays or verification of equivalence among assays using different amplification technologies (20). In short, the inherent limitations of the procedure make it inappropriate to use the procedure alone as a confirmatory test for the purpose of diagnosis. In this respect, the Western blot immunoassay, since its development in the late 1980s, has been the most commonly accepted confirmatory test. To many, it re-

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mains a gold standard for validating screening test results and provides valuable information for the diagnosis of an HIV infection (20, 23, 57, 87). In contrast to ELISAs or rapid tests that provide results reflecting the reactivities of antibodies to any or all of the antigens indiscriminately for screening purposes, Western blotting or line immunoassays with separated viral proteins immobilized on membrane testing strips generate specific information on the reactivities of antibodies to individual proteins (74). Such specific and detailed information can then be interpreted according to approved criteria set by the relevant authorities for validating the positive results of screening tests and hence ruling out those with false detection (15, 106). However, with reactivity specific to at least two of the proteins p24, gp41, and gp120/gp160 required for interpretation as a positive result, Western blotting from time to time generates reactivity profiles that are not compatible with either a positive or a negative interpretation and hence produces indeterminate results. Although these Western blot-indeterminate (WBi) results do not facilitate a final clinical decision, they often reflect the true serological statuses of the tested subjects, such as having acute HIV-1 or HIV-2 infections or possessing certain interfering factors. Hence, indeterminate results are more acceptable than erroneous HIV antibody-positive results by screening tests because the former indicates the right direction for further evaluation of the patients (63).

Nevertheless, WBi results are a real issue in managing testing laboratories and patients for HIV testing, especially at times of high occurrence. When indeterminate results are obtained, they hinder clinical decisions, create more work, take up more resources in laboratories, and cause inappropriate anxiety to patients or even undesirable consequences (73, 87). Although there have been various studies relating to the topic, new circumstances have arisen and new challenges have emerged, particularly with the introduction of fourth-generation ELISAs into the testing scheme. Hence, the intent of the present review is to provide an update to our understanding of this issue so that relevant countermeasures can be developed to overcome the challenges.

## CAUSES AND FREQUENCY OF WBi RESULTS

A Western blot for HIV-1 testing typically contains the following proteins: p17, p24, p31, p39, gp41, p51, p55, p66, and gp120/gp160. Most regulatory bodies rely on at least one of the envelope proteins (gp41 and gp120/gp160) with one of the core proteins (p17, p24, and p55) or one of the enzyme proteins (p31, p51, and p66) as the minimum criteria for interpretation of a positive result (15, 106). Incomplete banding profiles of tested strips, showing specific reactivity to any of the viral proteins not compatible with the approved criteria for a positive interpretation, are thus considered indeterminate. WBi results are neither new nor unique to HIV testing (49, 67, 75). In some earlier studies, researchers looked into various cohorts, including HIV initial-screen-positive subjects, healthy volunteers for vaccine trials, healthy blood donors who had negative initial-screen results, and their respective recipients (25, 30, 58, 104). While some of the indeterminate results might be at least partially related to variability in the performance of earlier products (30, 58), others were found to be fairly consistent and reproducible (25). Studying 20 enzyme immunoassay (EIA)-positive blood donors, Dock et al. (25) obtained consistent antibody reactivity (atypical results of Western blotting) throughout the follow-up period for 19 of the subjects, with only one case of seroconversion for HIV. Further, the study showed that 18 of these subjects were found to have reactivities specific to the core protein on the Western blot and 10 subjects were found with cross-reactivity to human T lymphotropic virus IV (HTLV-IV) (HIV-2) (25). The study thus demonstrated that indeterminate results were due not simply to product defects, but also to factors and conditions attributable to the test subjects. Based on the finding, Dock et al. suggested excluding individuals with EIA-positive results but atypical Western blot findings from the donor pool (25). On the other hand, indeterminate results were also found to exist even in a community with low prevalence with no apparent involvement of any risk factors for HIV (104). A separate study of donors with initial negative screening results revealed that WBi patterns were common in donors and their recipients and suggested that they were not correlated with the presence of HIV-1 or transmission of HIV-1 from donor to recipient (30).

Obviously, our concerns include both the true causes, such as factors/conditions associated with WBi results, and the appropriate approaches for handling the results for various cohorts, especially healthy blood donors from a low-prevalence community. Nuwayhid (64) provided a comprehensive summary of the associated conditions, but an update is overdue. Additional information available since that review should be included in our consideration of how to deal with various situations arising from WBi results. Hence, the attempt here is to include more current information to further clarify all the relevant causes. The following discussion and subdivision of the reported conditions and factors come from a practical perspective, in the hope of providing a clearer picture of the causes so that further appropriate measures can be developed for dealing with WBi (Table 1).

**WBi results associated with tested subjects.** Although various factors and conditions are recognized as being associated with WBi results in HIV testing, it is important to bear in mind that all atypical Western blots are the result of reactivity or cross-reactivity between the viral proteins and tested specimens. Hence, the serological statuses of test subjects from whom the specimens are obtained ultimately play an essential part in contributing to the indeterminate outcome. When apparent factors associated with assay conditions and the performance of kits can be excluded, WBi results denote to a great extent the immune statuses of the tested subjects, be it a true infection or association with other medical conditions. This was largely demonstrated by various reports correlating WBi results with individuals having true exposure to HIV or other retroviruses (6, 25, 50) or having other medical conditions (96). Even for individuals with no apparent infections, WBi results could still be an indication of subjects having various conditions, including possessing antibodies originating from passive transfer (25).

For HIV infection-related conditions, numerous studies demonstrated beyond doubt that at least a portion of WBi results were from early seroconversion, especially for high-risk individuals, and the incomplete patterns would eventually evolve into the full positive profile, showing great value for

TABLE 1. Causes of and conditions associated with WBi results

| Association | Status | Condition | Reference(s) |
|---|---|---|---|
| Subjects | HIV and related infections | Early seroconversion | 13, 25, 40, 65, 77 |
| | | Advanced AIDS | 6, 50 |
| | | HIV-2 infection | 25, 47 |
| | | Abortive infection | 31 |
| | | Infection with deletion strains | 69 |
| | | Immunosilent AIDS | 46 |
| | | Divergent HIV/simian immunodeficiency virus | 77 |
| | Other retroviral infection | HTLV infection | 25 |
| | | CAEV | 91 |
| | Other medical condition | Autoimmue diseases (systemic lupus erythematosus, primary Sjogren's syndrome) | 2, 89, 90, 96 |
| | | Leprosy | 44 |
| | | Malignancy | 96 |
| | | Elevated bilirubin, nucleoproteins, and rheumatoid factor | 7 |
| | | HLA | 3 |
| | No infection | Noninfected children born to infected mothers | 11 |
| | | Recipients of unscreened products | 30 |
| | | Participant vaccine trial | 45, 104 |
| | | Donors with consistent WBi results | 25, 26, 30, 104 |
| Kit design | | Host cell proteins | 20, 72, 73 |
| | | Interpretation criteria | 54, 87 |
| | | Optimizations/consistency of kits | 30, 58, 105 |
| Assay process | | Cross-contamination | 39, 62 |
| | | Test routine | 39 |
| | | Equipment | 62 |
| | | Sample treatment and hemolysis | 16, 32, 42 |

prognosis (13, 25, 40). Indeed, earlier studies had established the associations between atypical Western blot profiles and individuals having incomplete development of antibodies in the acute phase of infection (13, 25, 40), depletion of antibodies in the late stage of AIDS (6, 50), or infection with HIV-2 (25, 47). However, subsequent reports showed that there were more conditions associated with WBi results than previously suggested. Georgoulias et al. (31) reported that WBi in some low-risk blood donors could be an indication of abortive infection, whereas Rhodes et al. (69) offered an explanation for associating the WBi results with infections by deletion strains in identifying long-term survivors who received HIV-positive blood. Furthermore, Kopko et al. (46) documented that there were cases of "immunosilent infection" that would also bring about WBi results. Under the specific circumstances, it is noteworthy that the WBi result obtained for the "immunosilent infection" was due to *env*-related reactivity having only an anti-gp160 band (46) instead of the more commonly occurring anticore (p24) activity (5, 96). Separately, in a very recent report, Schaefer et al. (77) suggested yet another plausible cause for WBi results: individuals having been infected with divergent HIV or simian immunodeficiency virus. Although the study failed to present molecular evidence with all except one of the 70 WBi samples, it did show that the indeterminate result for the particular specimen was due to a recent infection with an HIV phylogenetically close to a recombinant viral strain (77).

Not unexpectedly, some of the WBi results were found to be associated with individuals having infections with HTLV or other retroviruses (25, 35). A recent study determined that there were homologous regions in the surface glycoprotein sequences of HIV and the corresponding nucleotides of the lentivirus caprine arthritis-encephalitis virus (CAEV) (91). The finding thus revealed the basis for some of the cross-reactivity and suggested that human contact with CAEV could be yet another source of false reactivity and indeterminate results. Although the atypical results of Western blotting obtained in the study were not the WBi results under discussion here in the strict sense, they demonstrated that the cross-reactivity of HIV proteins to associated antibodies against other lentiviruses occurred more broadly than originally revealed (91). Hence, it is not surprising to see the speculation of Hart et al. (35) that schizophrenia, schizophrenia spectrum disorder, and bipolar disorders might be associated with exposure to HIV-related retroviruses, based on the indeterminate results of anti-p24 and -p17 obtained from the subjects studied.

WBi results were also found to be associated with a variety of medical conditions or disorders other than HIV or retrovirus-related infections. Separate studies had established correlation of WBi results with systemic lupus erythematosus, rheumatoid factor, leprosy, post-measles virus infection, elevated bilirubin, polyclonal gammopathies, and hemodialysis (88, 89, 90, 98). However, a study by Urnovitz et al. (96) showed that, in addition to autoimmune diseases, malignancies, urologic disorders, sexually transmitted diseases, and multiple blood transfusions could also be responsible for the occurrence of WBi results. The study included serum samples from individuals having the above-mentioned conditions and requiring medical treatment or hospitalization and revealed a higher frequency of WBi results within the group: 36.7% in contrast to the 9.8% obtained with those from a HIV low-risk group. In

particular, the autoimmune disorders included in the study consisted of rheumatoid arthritis, Sjogren's syndrome, systemic lupus erythematosus, idiopathic thrombocytopenia purpura, myasthenia gravis, multiple sclerosis, and autoimmune hemolytic anemia, whereas the malignancies included chronic lymphocytic leukemia, breast cancer, lung cancer, colon cancer, Hodgkin's disease, and multiple myeloma. The urologic disorders included acute glomerular nephritis, acute tubular necrosis, acute renal failure, chronic renal failure, and nephrotic syndrome (96). Although the study did not pinpoint which of the particular disorders contributed directly to the WBi results, it resembled HIV testing in hospital settings to a great extent and certainly highlighted the significance of other medical disorders in contributing to the WBi results (96). It is noteworthy that the WBi band patterns did not appear at random in that study; they occurred more commonly for the core antigens, particularly p24 (96). Concerning the basis for the cross-reactivity resulting in WBi with the particular cohorts, documented explanations include the following: (i) autoantibodies in systemic lupus erythematosus patients could cross-react with p24 (88, 89), (ii) p24-reactive antibodies in patients with systemic diseases were directed to viral epitopes (43), (iii) anti-HLA sera contained cross-reactive antibodies to HIV viral proteins (3), (iv) mycobacterial cell wall antigens might share common epitopes with HIV (44), and (v) transplantation antigen and/or autoantibody formation in hemodialysis patients could cause abnormal immune reactivity (98).

Unlike the above-mentioned associations, there were no immediate explanations for WBi results from individuals among healthy blood donors in a low-prevalence community who had no apparent infection. Although there were observations that associated WBi with recipients of unscreened blood products with no apparent seroconversions or infections (12, 30, 48) or loss of antibodies acquired from HIV-infected mothers in seroreverted children (11), none of these examples could readily explain some of the persistent indeterminate results in low-prevalence cohorts. Various long-term follow-up studies revealed no evidence of HIV infection of healthy individuals who had persistent WBi results and hence concluded that WBi results were exceedingly common in testing low-risk donors (26, 30, 37, 38, 41). The CDC has recognized the existence of WBi results in low-prevalence cohorts and has implemented guidelines for designating those individuals negative when no risk factors were involved and no changes in immunoreactivity profiles were obtained with follow-up testing (17). While the recommendations address the practical issues, they fail to address the fact that we lack an answer to the fundamental question of what causes the phenomenon. In this respect, the identification of homologies between HIV type O *env* sequences and 14 different human chromosomes (96) is noteworthy, as is the fact that sera from healthy individuals often contain antibodies to the interspecies antigens of mammalian type C retroviruses (97). It may not be unreasonable to hypothesize the involvement of endogenous retroelements, and further studies in the area may prove rewarding.

**WBi results contributed by test kits.** It is much less ambiguous that WBi results could also be due to batch-to-batch variation of test kits, especially when the reproducibility of results has been in doubt (58). Similarly, the performance of kits should be questioned if a sudden change in the frequency of WBi results occurs with no other apparent alterations in the testing procedures or cohorts (62). However, under such circumstances, it is critical to determine whether the WBi results obtained are due to inherent shortcomings of kits or erroneous assay handling (see below), because the manifestations of the two often resemble one another greatly. The inherent shortcomings of test kits could be derived from quality-related issues in material usage, in optimization, or in production of the assay. For example, if nonviral proteins derived from the host cells (in which the virus was cultured) were not removed adequately, some cross-reactivity could result when antibodies reactive to the proteins are present (20, 73). These nonviral proteins could contribute to the WBi result or add to the confusion in interpretation of the results (20). On the other hand, the criteria employed for interpretation of testing results could also affect the ultimate conclusion drawn. One example of this nature was from a report from the 1980s, when an ELISA-seropositive sample was apparently confirmed as positive (subsequently verified as FP) even though it had reactivity only to a p52 protein by Western blotting (73). Obviously, such a result would be interpreted as indeterminate by today's criteria. In a study by Mahe et al. (54), the frequency of WBi results was significantly reduced by modifying the interpretation criteria, even though the assumption on which the modification was based appeared debatable. Furthermore, Syed Iqbal et al. (87) were able to show that applying different criteria recommended by the respective authorities was one of the driving factors determining the frequency of the WBi results for at least one of the commercial kits tested.

**WBi results due to assay processes.** Other, less frequent, and sometimes overlooked causes for WBi results were related to assay processes. As demonstrated by Ngan et al. (62), the increase of WBi results from 10.5% to 12.2% could easily be attributed to the quality of the product, but careful observations led to the discovery of cross-contamination due to carryover of positive sera from adjacent wells of the tray used for the semiautomated machine. Other, apparently trivial but actually significant factors contributing to the occurrence of WBi, such as sample and reagent dilution, incubation time, and incomplete washing or incubation at temperatures greater than 30°C, were highlighted by manufacturers, with corresponding guidelines for troubleshooting. Sometimes, it was much easier to overlook than to detect inadequate washing, especially when automation was involved, unless a total failure became apparent. In this respect, it is particularly relevant to include the findings of Jamjoom et al. (39) in our discussion. With the introduction of testing samples with high optical density and low optical density separately and additional steps for rinsing the suction apparatus after each sample to prevent backflow aerosol contamination, a significant reduction of indeterminate results from the initial 20% in 1994 to 8.3% in 1996 was achieved (39). Obviously, only careful analyses including all relevant factors would enable effective determination of whether problems with a specific incident were due to inherent shortcomings of kits or assay processes.

In addition, it is also noteworthy that the CDC considered specimen preparation another relevant factor and in fact cau-

TABLE 2. Frequency of WBi results with different cohorts from different territories

| Country or region | Cohort | Population total (*n*) | No. screen positive (no. negative) | No. of WBi results (%) | Reference |
|---|---|---|---|---|---|
| United States | Blood donor | 500,000 | 1,100 | 160 (14.5) | 80 |
| United States | Blood donor | 3,270,000 | 2,660 | 1,207 (45.4) | 27 |
| Peru | General population | 1,363 | 35 | 13 (37.1) | 19 |
| Chile | Donor and risk group | 600,000 | 4,956 | 409 (8.3) | 68 |
| India | Blood donor | 39,784 | 44 | 5 (11.4) | 92 |
| Nigeria | Blood donor | 500 | 186 | 55 (29.6) | 28 |
| Ethiopia | Volunteers[a] | 12,124 | 1,437 (1,475)[c] | 91 (6.3)/31 (2.1)[c,e] | 57 |
| Brazil | Rejected donor | NA[b] | 210[d] | 6 (2.9) | 4 |
| Central Africa | High-risk group | 1,998 | 1,065 | 367 (34.5) | 5 |
| United States | High-risk group | NA | 371 | 123 (33.2) | 96 |
| United States | Other disorders | NA | 199 | 73 (36.7) | 96 |
| Saudi Arabia | Hospital/health center cohorts | NA | 2,849 | 444 (15.6) | 39 |
| Brazil | Pregnant women | 9,786 | 105 | 11 (10.5) | 21 |
| Cameroon | Pregnant women | 859 | 163 | 13 (8.0) | 33 |

[a] Participants in Ethio-Netherlands AIDS Research Project.
[b] NA, not available.
[c] Only 1,475 of the 10,687 screened negative were included in Western blot testing.
[d] Selected cohort; no association was made with the initial population screened.
[e] The two values are number of WBi results among those who screened positive/number of WBi results among those who screened negative, with percentages for each given parenthetically.

tioned users about the effects of heat inactivation of samples prior to antibody testing for HIV (16). However, the interference of conventional heat treatment at 56°C for 30 min (32) appeared to be a much less significant factor, at least with kits of recent manufacture (99).

Another matter of concern relating to processes is the impact of the algorithms employed in testing. However, this important issue warrants a section of its own and is discussed separately below.

**Frequency of WBi results.** Understandably, the frequency of WBi results varied from kit to kit, from territory to territory, from population to population, and from algorithm to algorithm. In a very recent study, Syed Iqbal et al. (87) were able to associate the different frequencies of WBi results at 7% to 25.6% with different kits (even though the main focus of the study was on the impacts of criteria on result interpretation). However, Urnovitz et al. (96) demonstrated quite clearly that different cohorts would yield different numbers of WBi results, with indeterminate detection at 9.8%, 33.2%, and 36.7%, respectively, for the HIV low-risk cohort, the HIV high-risk cohort, and the cohort with other medical conditions. It appeared that the frequency was somewhat associated with the risk of HIV infection and other medical conditions, predominately autoimmune-related disorders (96). However, such differences appeared less obvious in a brief examination of other publications covering different territories and cohorts. As shown in Table 2, there were great variations in the frequency of occurrence of WBi results, from 6% to almost 50%, even within the same cohort of blood donors. Although those data were not generated within one study and hence are not strictly comparable, they do provide some indication of the complexity of the issue. It appears highly debatable that the frequency of WBi results could be simply attributed to any one of the factors discussed above or to the differences among the cohorts. In this respect, it is important to recognize the findings by Dodd and Stramer that the number of WBi results was proportional to the number of samples tested rather than the prevalence of infection, based on their studies involving 3.27 million subjects (27). In fact, they suggested that the use of a primary screening test with poor specificity would be the ultimate cause of WBi results (27). This was very strongly supported by the subsequent findings of Prince and Gross (67), who illustrated the impact of initial screening on the efficiency of Western blot testing for HTLV and the importance of adherence to the recommended algorithm for overcoming a similar WBi problem. Specific to HIV testing, a clear demonstration was provided in the study by Huang et al. (36). The screen algorithm comprising repeat testing with one ELISA instead of the ELISA in combination with a more specific agglutination test generated not only more FPs (143 versus 8), but also more WBi results (88 [36.5%] versus 2 [10%]) with the same testing cohort of 281 samples (36). Separately, it is also noteworthy that 13 WBi results were fully resolved when an additional screen assay was introduced into the testing algorithm prior to the Western blot confirmation (33).

On the other hand, the frequency of bands for individual viral proteins among the WBi patterns appeared not to be as random an event as indicated above. Notably, the band for reactivity to p24 viral protein was the most frequently occurring band among all the WBi patterns regardless of cohorts or study settings (5, 10, 96). Studying 84 blood donors with repeated indeterminate results, Carneiro-Proietti et al. (10) obtained a frequency for the anti-p24 band of 91.6%. Although this was found at a more moderate level of 74% in a separate study of Urnovitz et al. (96), the anti-p24 band remained predominant among all the other antiviral markers, with the next highest reaching only 16% for an HIV-1 low-risk group. Similarly, the frequency of the anti-p24 band was at a lower level of 60% among the WBi patterns obtained with an HIV-1 high-risk group, but it was again the most frequently occurring marker, with the next most frequent reaching the rate of 21.9%. In contrast, the anti-gp120/gp160 bands appeared much less frequently in general, and occurrences were more likely to be associated with the HIV-1 high-risk group (96) or with individuals having silent infection (46).

## STANDARD AND ALTERNATIVE ALGORITHMS FOR CONFIRMATION OF HIV INFECTION AND THEIR CONSEQUENCES

**Standard testing algorithm.** The current standard testing algorithm for diagnosis of HIV infection can be traced back to practices in the mid- to late 1980s and was derived from the CDC guidelines (14, 17). The algorithm requires a sequence of tests starting with repeatedly reactive EIA and a subsequent confirmation of Western blotting (14). Consequently, a sample can be considered positive for HIV only if it tests initially positive by an EIA and subsequently positive by a repeat test in duplicate with the same EIA and is confirmed positive by a supplemental test, such as Western blotting or, less frequently, immunofluorescence assay (17). The algorithm was therefore designed to reduce the chance of an FP, and accordingly, the purpose of Western blotting was to achieve the objective of ruling out FP results. Inherently, the interpretation guideline for Western blots was focused very much on the identification of positive results with relatively little consideration of indeterminate results. Nevertheless, the algorithm itself, with the serial tests prior to confirmation, when adhered to closely could reduce the number of FPs derived from errors of operational origin. While the indeterminate result was posed as an issue for clarification, there was also concern about the cost-effectiveness of the algorithm. Consequently, alternative strategies for HIV testing were proposed and adopted by UNAIDS and WHO (94, 95, 107).

**Alternative algorithms.** Based on the updated guideline of UNAIDS/WHO (107), three alternative testing algorithms were recommended in dealing with different scenarios for HIV testing. These algorithms, designated strategies I, II, and III, were designed to eliminate the use of confirmatory tests, such as Western blotting, and hence reduce the testing costs for resource-poor countries. In particular, strategy I (requiring only one test) is intended for use in diagnostic testing in populations with an HIV prevalence of >30% among persons with clinical signs and symptoms of HIV infection and in blood screening and surveillance testing in populations with an HIV prevalence of >10%. Strategy II (requiring two tests) is a moderate approach for use in surveillance testing in populations with an HIV prevalence of ≤10% and in diagnostic testing in populations with a prevalence of ≤30% among persons with clinical signs and symptoms of HIV infection or >10% among asymptomatic persons. The most stringent approach is strategy III (requiring three tests utilizing different antigens and/or different test principles) for diagnostic testing in populations with an HIV prevalence of ≤10% among asymptomatic persons.

While these approaches might have proven cost-effective in some resource-lacking countries, with different cohorts, or even with different types of specimens (24, 33, 56, 82), the impacts of the recommendations on diagnostic and detection outcomes are beyond immediate comprehension. In pursuing the recommended strategies, various combinations of test kits were reviewed and ultimately adopted in routine HIV testing. Notably, the approaches included using combinations such as ELISA and Western blotting, ELISA and rapid test, or rapid test and rapid test either consecutively or simultaneously. Nonetheless, all combinations were found to have inherent shortcomings (33, 56, 63, 71, 82). As discovered by Ngan et al., (63) in using three rapid tests for confirmation of 2,256 referral samples, almost half of those positive (28 of 61) by all three tests proved to have indeterminate results by Western blotting. In this case, the uncertainty of WBi results was surely more acceptable than FP results, as the WBi results indicated the right direction for further evaluation (63). Notably, this was not an isolated incident and was further supported by various findings of recent studies (33, 82). In a study involving over 800 subjects, Granade et al. (33) designed different approaches of both serial testing (two consecutive tests) and parallel testing (two simultaneous tests) for their alternative algorithms, using a third test for resolving any discordance. Although they were able to avoid any uncertainty of WBi results with their serial and parallel testing, both approaches produced FP results (33). Similarly, in yet another study of 3,500 samples, the rapid tests under the strategy III approach produced 33 FPs out of the 507 samples identified (82). These examples amply illustrated the impacts of the algorithms utilized on the detection outcomes. Indeed, even the most stringent strategy III for confirmation was not trouble free and hence should be treated with prudence when followed (63). The dilemma, at least in theory, appeared to be whether one wants to face the uncertainty of WBi or "certainty" with a proportion of FP results, in addition to the cost-effectiveness consideration. In reality, alternative algorithms did not automatically lead to an indeterminate-free situation. Using two rapid tests in serial testing following the strategy II approach, Rouet et al. actually produced 113 indeterminate cases out of the 1,293 initial-positive cases, which could be resolved only by additional tests using a supplemental ELISA (71). In fact, an earlier study justifying the cost-effectiveness of alternative algorithms managed to avoid the complication only by excluding the group of samples with WBi results from its data analysis (56).

The problem appeared to be that the different algorithms/strategies were intended for the respective purposes, i.e., transfusion/transplant safety surveillance, surveillance diagnosis, and, ultimately, confirmatory diagnosis of HIV infection. However, the outcome of utilizing different algorithms/strategies could hardly be treated in accordance with the defined intents. It is understandable that users of strategy I, II, or III would find it equally hard to accept any uncertain or erroneous outcome, even though different strategies are known to produce different consequences. Hence, in reality, a blood-screening facility adopting an algorithm using one screening assay followed by one supplemental Western blot test would find it hard to deal with WBi resultseven if such WBi results could be resolved by implementing one additional screening test. In this respect, the finding of Granade et al. that 13 of the indeterminates generated by the one screening assay and one Western blot combination approach were fully resolved by the two screening assays with one Western blot option is interesting (33). Although there were additional variables in terms of the kits used, this example nevertheless illustrated the different consequences using a strategy II approach and the standard algorithm. The finding of Granade et al. thus demonstrated the limitation of Western blotting, and users should be reminded of the fact that this supplemental test was not designed for screening (20). Indeed, additional WBi samples could be generated from a

screened negative population if the cohort was also tested by Western blotting (57).

## NEW CHALLENGES AND FURTHER CONSIDERATIONS

**Fourth-generation ELISA and WBi.** Abundant literature on fourth-generation ELISAs exists, but relatively less was known about the impact on the incidence of WBi or overall testing with the introduction of this technology into the HIV testing algorithms (34, 52, 53, 76, 100, 101, 103). Most of the studies appeared to have centered on the performance of the technology in terms of closing the detection window, and hence, little attention was given to the possible consequences of the addition of the technology to the existing test algorithms. In theory, the potential for nonspecific reactivity with fourth-generation ELISAs should be higher than that of the third generation because the former combine two different test principles in one assay (101). In practice, it appeared inconsistent and debatable whether fourth-generation ELISAs were indeed more inclined to have nonspecificity issues than their third-generation counterparts. While Weber et al. (101) reported improved performance of the fourth generation over the third, Ly et al. (52) demonstrated exactly the opposite, with a better specificity of 99.86% for the third-generation ELISA in contrast to the 99.51% for the fourth-generation test. Although the difference between the two percentages might appear small, in reality, it was equivalent to an addition of over 100 cases of FP with 29,657 cases tested during a 9-month survey in a cohort with a positive prevalence rate of 1.52% (52). In fact, of the 613 positives identified by the fourth-generation ELISA, 143 (23.3%) were found to be FP, in contrast to 42 FP (8.5%) out of the 495 positives identified by the third-generation ELISA (52). Hence, it is not far-fetched to say that an increased rate of WBi could also be associated with using the fourth-generation ELISA, even though none has so far been reported with existing studies focused on evaluation. In reality, an increase in the incidence of WBi seen in customer feedback, especially those with only anti-p24 reactivity, were more often than not associated with the use of fourth-generation ELISAs as the primary test (unpublished data). This appeared explainable, at least in theory, by the fact that only fourth-generation ELISAs had the capacity to detect additional samples with reactivities specific to or associated with viral p24 antigen. Nevertheless, as pointed out by Dodd and Stramer (27), the performance of a primary specificity test was directly proportional to the number of WBi results obtained.

Although WHO and the CDC have acknowledged the existence of the fourth-generation ELISA, neither appeared to have taken the unique requirements of the technology into consideration in the latest update on recommendations for their testing algorithms (18, 107). It is obvious that the simultaneous detection of antigen and antibody by fourth-generation ELISAs will result in identification of samples containing either antibody, antigen, or both. Consequently, confirmation of reactivity generated by these ELISAs would require a special algorithm consisting of two supplemental tests different from those approaches currently recommended. In particular, an antibody test, such as Western blotting or immunoblotting, is needed for verification of the anti-HIV portion of the reactivity, whereas an antigen test or a nucleic acid-based assay is needed for the p24 antigen portion of the reactivity (52). Consequently, Brust et al. (8) proposed a confirmatory strategy that repeat positives produced by fourth-generation ELISAs should be confirmed in parallel with an antigen-testing and an antibody-testing approach, with each route consisting of a screen test and a supplemental test. This algorithm would keep the residual risk to an absolute minimum, but the excessive use of tests is also obvious. Based on the data of Ly et al. (52) obtained from real laboratory settings, it appeared more cost-effective to use an antibody test for the overall confirmation, since over 95% of the reactivity by the fourth-generation ELISA was anti-HIV related and verifiable by Western blotting. However, a further supplemental test using either a dedicated p24 antigen test or a nucleic acid assay appeared unavoidable for verification of the reactivity related to p24 antigen. Correspondingly, WBi results generated by a fourth-generation ELISA as a primary test in low-prevalence cohorts should be additionally tested with an antigen test or molecularly based test prior to the recommendation of follow-up sampling, unless other factors, such as clinical evidence, pointed to the possibility of seroconversion.

**Differentiation of true infection from nonspecific reaction.** The prime concern with WBi result is obviously the derived uncertainty in decision making for diagnosis, and hence, the ultimate objective in managing WBi results is to enable the differentiation of a true infection from nonspecific reactions. As is understood, anti-HIV antibodies, once developed, persist for a lifetime (107). Furthermore, as reviewed above, incomplete Western blot profiles for individuals having a true HIV infection would be associated only with a primary infection at the time of seroconversion or at the late stage of AIDS. Consequently, our main focus for handling WBi results is on how to identify any true case of seroconversion among all the indeterminates, rather than with monitoring the progression of the disease, as the latter involves no decision making relating to diagnosis. With this focus in mind, it is critical to understand the dynamics of various viral or antiviral markers against the time course in individuals after an exposure to HIV. Furthermore, it is essential that we recognize the subtle difference between the "window period" and "seroconversion." While the former refers to the duration from exposure to the time when respective (viral or antiviral) markers first become detectable, the latter denotes the process of development of antibodies to a specific pathogen. In this respect, there is no existing terminology covering the interval immediately after the window period but prior to the full manifestation of all anti-HIV markers by Western blotting, which in fact is most relevant to our discussion of WBi. Perhaps it is appropriate that we define the period as the "WBi interval," as it is associated only with the Western blot technology and no equivalent progression exists for all viral markers, such as RNA or p24 antigen. Based on the model established by Fiebig et al. (29) using plasma samples from donors, the window periods for HIV RNA, p24 antigen, and anti-HIV antibody are 12 days, 17 days, and 22 days, respectively. Although any baseline established would obviously depend very much on the sensitivity of the kits used for the construction, the finding of Fiebig et al. was not a significant deviation from that of a previous model by Busch and Satten (9). The two exceptions included in the study by Busch and Satten (with a >6-month window period) were

associated with health care workers with needle stick-acquired infections. This is not the most common route of HIV exposure, and hence, application of these results should be treated with caution. Nevertheless, our concern with managing the WBi results is much less relevant with the window period but more so with the "WBi interval." This is because, with a WBi result, the status of a tested individual would have already progressed beyond the window period and to the late stage of seroconversion—the "WBi interval"—if a true HIV exposure occurred earlier. In view of this, the proposal by Fiebig et al. (29) of six laboratory stages of primary HIV infection is most applicable to our discussion here. The six stages are as follows: stage I, RNA assay reactive only; stage II, RNA assay and p24 antigen assay positive; stage III, RNA, p24 antigen assay, and EIA positive but Western blot negative; stage IV, like stage III, but with WBi results; stage V, like stage IV but with WB positive lacking p31 reactivity; stage VI, like stage V but with full Western blot reactivity, including a p31 band. It is noteworthy that the projected duration for each of the stages I to IV is brief, lasting on average only 3 to 5 days (29). Based on these findings, two important features emerged. First, although the window period for WB could be as long as 22 days, once initiated, the progression (the "WBi interval" covering the period of stage IV) to a full profile took no longer than 8 days for true cases of infection. Second, during this "WBi interval," when Western blotting was indeterminate, reactivity detectable by a p24 antigen assay was reduced to a relatively low level but that detectable by an RNA assay was not. In fact, the HIV RNA level peaked at stage III but was sustained at a detectable level well beyond stage VI (29, 61). Some long-range follow-up studies also showed that high levels of HIV RNA were detectable in plasma prior to seropositivity and were maintained thereafter beyond 30 months in both vertically infected infants and heterosexually infected adults (55, 79). Therefore, it is adequate to conclude that although repeat antibody test results can remain negative for months after exposure with some extreme cases, no true case of infection with WBi lasts for weeks without being accompanied by at least one other detectable viral marker. This was indeed supported by a separate study involving 436 patients with primary infection, while none of the 13 cases with WBi results was found to be undetectable by the HIV RNA test (22). Conversely, no true case of seroconversion was evident in follow-up studies after a confirmation of negative results by PCR methods among the 26 samples from 23 individuals having screened positive but with WBi results (78). Consequently, it is possible to use molecular testing for an early resolution of WBi results as an alternative to the current approach with a follow-up test after 1 month (17). It is appropriate to propose that a WBi result can be treated as negative if it is not accompanied by positive detection using a molecular test, in particular, an RNA test with primer sets covering HIV-1/2/O and meeting the sensitivity of today's standard. This is particularly relevant with WBi samples derived from a test algorithm using fourth-generation ELISAs as a primary test for healthy blood donors without any risk factors involved. If the reactivity of a positive result by a fourth-generation ELISA accompanied by a WBi result cannot be confirmed by another antibody-only test, it is more likely that it will be verifiable by an antigen test or molecularly based test if the reactivity was due to a true infection in the first place. In other words, a negative outcome is indicated if the results were not supported by, or accompanied by, a positive result by an antigen test or a molecularly based assay, especially an RNA assay, with a performance sensitivity meeting today's standard.

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# ATTACHMENT 19

# COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test

**FOR *IN VITRO* DIAGNOSTIC USE.**

| | | | |
|---|---|---|---|
| COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test | HIMCAP | 48 Tests | P/N: 03542998 190 |
| COBAS® AmpliPrep/COBAS® TaqMan® Wash Reagent | PG WR | 5.1 Liters | P/N: 03587797 190 |

## INTENDED USE

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is an *in vitro* nucleic acid amplification test for the quantitation of Human Immunodeficiency Virus Type 1 (HIV-1) RNA in human plasma using the COBAS® AmpliPrep Instrument for automated specimen processing and COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer for automated amplification and detection.

This test is intended for use in conjunction with clinical presentation and other laboratory markers of disease progress for the clinical management of HIV-1 infected patients. The Test can be used to assess patient prognosis by measuring the baseline HIV-1 RNA level or to monitor the effects of antiretroviral therapy by measuring changes in EDTA plasma HIV-1 RNA levels during the course of antiretroviral treatment.

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is not intended for use as a screening test for the presence of HIV-1 in blood or blood products or as a diagnostic test to confirm the presence of HIV-1 infection.

The test can quantitate HIV-1 RNA over the range of 48 - 10,000,000 copies/mL. One copy of HIV-1 RNA is equivalent to 1.7 ± 0.1 International Units (IU) based on the WHO 1st International Standard for HIV-1 RNA for Nucleic Acid-Based Techniques (NAT) (NIBSC 97/656).

## SUMMARY AND EXPLANATION OF THE TEST

Human Immunodeficiency Virus (HIV) is the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS)[1-3]. HIV infection can be transmitted by sexual contact, exposure to infected blood or blood products, or by an infected mother to the fetus[4]. Within three to six weeks of exposure to HIV, infected individuals generally develop a brief, acute syndrome characterized by flu-like symptoms and associated with high levels of viremia in the peripheral blood[5-8]. In most infected individuals this is followed by an HIV-specific immune response and a decline of plasma viremia, usually within four to six weeks of the onset of symptoms[9,10]. After seroconversion, infected individuals typically enter a clinically stable, asymptomatic phase that can last for years[11-13]. The asymptomatic period is characterized by persistent, low level plasma viremia[14] and a gradual depletion of $CD4^+$ T lymphocytes, leading to severe immunodeficiency, multiple opportunistic infections, malignancies and death[15]. Although virus levels in the peripheral blood are relatively low during the asymptomatic phase of the infection, virus replication and clearance appear to be dynamic processes in which high rates of virus production and infection of $CD4^+$ cells are balanced by equally high rates of virus clearance, death of infected cells and replenishment of $CD4^+$ cells, resulting in relatively stable levels of both plasma viremia and $CD4^+$ cells[16-18].

Quantitative measurements of HIV viremia in the peripheral blood have shown that higher virus levels may be correlated with increased risk of clinical progression of HIV disease, and that reductions in plasma virus levels may be associated with decreased risk of clinical progression[19-21]. Virus levels in the peripheral blood can be quantitated by measurement of the HIV p24 antigen in serum, by quantitative culture of HIV from plasma, or by direct measurement of viral RNA in plasma using nucleic acid amplification or signal amplification technologies[22-26].

p24 antigen is the principal core protein of HIV and is found in serum either free or bound by anti-p24 antibody. Free p24 antigen can be measured with commercially available enzyme immunoassays (EIA), although the usefulness of p24 antigen as a marker of viral load is limited since the antigen is detectable in only 20% of asymptomatic patients and 40-50% of symptomatic patients. Procedures to dissociate antigen-antibody complexes improve the sensitivity of the p24 antigen tests, but the viral protein remains undetectable in most asymptomatic patients[22].

Infectious HIV in plasma can be cultured by inoculation into activated peripheral blood mononuclear cells (PBMC) from normal donors. Quantitation is achieved by inoculating PBMC with serial dilutions of the plasma specimen. Quantitative culture has limited utility for monitoring virus levels in infected individuals since only a small fraction of virus particles is infectious *in vitro*. Infectious virus is often undetectable in asymptomatic individuals[22].

HIV RNA in plasma can be quantitated by nucleic acid amplification technologies, such as the Polymerase Chain Reaction (PCR)[27-29]. The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test uses PCR technology to achieve high sensitivity and dynamic range for the quantitative detection of HIV-1 RNA in EDTA anti-coagulated plasma.

## PRINCIPLES OF THE PROCEDURE

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is a nucleic acid amplification test for the quantitation of Human Immunodeficiency Virus Type 1 (HIV-1) RNA in human plasma. Specimen preparation is automated using the COBAS® AmpliPrep Instrument with amplification and detection automated using the COBAS® TaqMan® Analyzer or the COBAS® TaqMan® 48 Analyzer.

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is based on three major processes: (1) specimen preparation to isolate HIV-1 RNA; (2) reverse transcription of the target RNA to generate complementary DNA (cDNA), and (3) simultaneous PCR amplification of target cDNA and detection of cleaved dual-labeled oligonucleotide probe specific to the target.

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test permits automated specimen preparation followed by automated reverse transcription, PCR amplification and detection of HIV-1 target RNA and HIV-1 Quantitation Standard (QS) Armored RNA. The Master Mix reagent contains primers and probes specific for both HIV-1 RNA and HIV-1 QS RNA. The Master Mix has been developed to ensure equivalent quantitation of group M subtypes of HIV-1. The detection of amplified DNA is performed using a target-specific and a QS-specific dual-labeled oligonucleotide probe that permit independent identification of HIV-1 amplicon and HIV-1 QS amplicon.

The quantitation of HIV-1 viral RNA is performed using the HIV-1 QS. It compensates for effects of inhibition and controls the preparation and amplification processes, allowing a more accurate quantitation of HIV-1 RNA in each specimen. The HIV-1 QS is a non-infectious Armored RNA construct that contains HIV sequences with identical primer binding sites as the HIV-1 target RNA and a unique probe binding region that allows HIV-1 QS amplicon to be distinguished from HIV-1 target amplicon.

The HIV-1 QS is added to each specimen at a known copy number and is carried through the specimen preparation, reverse transcription, PCR amplification and detection steps of cleaved dual-labeled oligonucleotide detection probes. The COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer calculates the HIV-1 RNA concentration in the test specimens by comparing the HIV-1 signal to the HIV-1 QS signal for each specimen and control.

### Target Selection

Selection of the target RNA sequence for HIV-1 depends on identification of regions within the HIV-1 genome that show maximum sequence conservation among the various group M HIV-1 subtypes. Generic silica based specimen preparation is used to capture the HIV-1 RNA and HIV-1 QS RNA and defined oligonucleotides are used as primers in amplification of the HIV-1 RNA and HIV-1 QS RNA. A target-specific and a QS-specific dual-labeled oligonucleotide probe permit independent identification of HIV-1 amplicon and HIV-1 QS amplicon. Accordingly, the appropriate selection of the primers and the dual-labeled oligonucleotide probe is critical to the ability of the test to amplify and detect the HIV-1 subtypes. The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test uses reverse transcription and PCR amplification primers that define a sequence within the highly conserved region of the HIV-1 *gag* gene[32]. The gag region encodes the group-specific antigens or core structural proteins of the virion. The nucleotide sequence of the primers has been optimized to yield comparable amplification of group M subtypes of HIV-1.

### Specimen Preparation

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test utilizes automated specimen preparation on the COBAS® AmpliPrep Instrument by a generic silica-based capture technique. The procedure processes 850 µL of plasma. The HIV-1 virus particles are lysed by incubation at elevated temperature with a protease and chaotropic lysis/binding buffer that releases nucleic acids and protects the released HIV-1 RNA from RNases in plasma. Protease and a known number of HIV-1 QS Armored RNA molecules are introduced into each specimen along with the lysis reagent and magnetic glass particles. Subsequently, the mixture is incubated and the HIV-1 RNA and HIV-1 QS RNA are bound to the surface of the magnetic glass particles. Unbound substances, such as salts, proteins and other cellular impurities, are removed by washing the magnetic glass particles. After separating the magnetic glass particles and completing the washing steps, the adsorbed nucleic acids are eluted at elevated temperature with an aqueous solution. The processed specimen, containing the magnetic glass particles as well as released HIV-1 RNA and HIV-1 QS RNA, is added to the amplification mixture and transferred to the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer. The HIV-1 target RNA and the HIV-1 QS RNA are then reverse transcribed, amplified and simultaneously

detected by cleavage of a target-specific and a QS-specific dual-labeled oligonucleotide probe.

**Reverse Transcription and PCR Amplification**

The reverse transcription and PCR amplification reaction is performed with the thermostable recombinant enzyme *Thermus specie* DNA Polymerase (Z05). In the presence of manganese ($Mn^{2+}$) and under the appropriate buffer conditions, Z05 has both reverse transcriptase and DNA polymerase activity[30,31]. This allows both reverse transcription and PCR amplification to occur together with real-time detection of the amplicon.

Processed specimens are added to the amplification mixture in amplification tubes (K-tubes) in which both reverse transcription and PCR amplification occur. The reaction mixture is heated to allow a downstream primer to anneal specifically to the HIV-1 target RNA and to the HIV-1 QS RNA. In the presence of $Mn^{2+}$ and excess deoxynucleotide triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine and deoxythymidine triphosphates, Z05 polymerase extends the annealed primers forming a DNA strand complementary to the RNA target.

Target Amplification

Following reverse transcription of the HIV-1 target RNA and the HIV-1 QS RNA, the Thermal Cycler in the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer heats the reaction mixture to denature the RNA:cDNA hybrid and to expose the specific primer target sequences. As the mixture cools, the primers anneal to the target DNA. The thermostable *Thermus specie* Z05 DNA Polymerase (Z05) in the presence of $Mn^{2+}$ and excess deoxynucleotide triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine and deoxythymidine triphosphates, extends the annealed primers along the target template to produce a double-stranded DNA molecule termed an amplicon. The COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer automatically repeats this process for a designated number of cycles, with each cycle intended to double the amount of amplicon DNA. The required number of cycles is preprogrammed into the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer. Amplification occurs only in the region of the HIV-1 genome between the primers; the entire HIV-1 genome is not amplified.

Selective Amplification

Selective amplification of target nucleic acid from the specimen is achieved in the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test by the use of AmpErase (uracil-N-glycosylase) enzyme and deoxyuridine triphosphate (dUTP). The AmpErase enzyme recognizes and catalyzes the destruction of DNA strands containing deoxyuridine[33], but not DNA containing deoxythymidine. Deoxyuridine is not present in naturally occurring DNA, but is always present in amplicon due to the use of deoxyuridine triphosphate as one of the dNTPs in the Master Mix reagent; therefore, only amplicon contains deoxyuridine. Deoxyuridine renders contaminating amplicon susceptible to destruction by the AmpErase enzyme prior to amplification of the target DNA. Also, any nonspecific product formed after initial activation of the Master Mix by manganese is destroyed by the AmpErase enzyme. The AmpErase enzyme, which is included in the Master Mix reagent, catalyzes the cleavage of deoxyuridine-containing DNA at the deoxyuridine residues by opening the deoxyribose chain at the C1-position. When heated in the first thermal cycling step, the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. The AmpErase enzyme remains inactive for a prolonged period of time once exposed to temperatures above 55°C, i.e. throughout the thermal cycling steps, and therefore does not destroy target amplicon formed after PCR.

**Detection of PCR Products in a COBAS® TaqMan® Test**

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test utilizes real-time[34,35] PCR technology. The use of dual-labeled fluorescent probes allows for real-time detection of PCR product accumulation by monitoring of the emission intensity of fluorescent reporter dyes released during the amplification process. The probes consist of HIV-1 and HIV-1 QS-specific oligonucleotide probes with a reporter dye and a quencher dye. In the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, the HIV-1 and HIV-1 QS probes are labeled with different fluorescent reporter dyes. When these probes are intact, the fluorescence of the reporter dye is suppressed by the proximity of the quencher dye due to Förster-type energy transfer effects. During PCR, the probe hybridizes to a target sequence and is cleaved by the 5' $\rightarrow$ 3' nuclease activity of the thermostable Z05 DNA polymerase. Once the reporter and quencher dyes are released and separated, quenching no longer occurs, and the fluorescent activity of the reporter dye is increased. The amplification of HIV-1 RNA and HIV-1 QS RNA are measured independently at different wavelengths. This process is repeated for a designated number of cycles, each cycle effectively increasing the emission intensity of the individual reporter dyes, permitting independent identification of HIV-1 RNA and HIV-1 QS RNA. The PCR cycle where a growth curve starts exponential growth is related to the amount of starting material at the beginning of the PCR.

## Fundamentals of COBAS® TaqMan® Test Quantitation

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is inherently quantitative over a very wide dynamic range since the monitoring of amplicon is performed during the exponential phase of amplification. The higher the HIV-1 titer of a specimen, the earlier the fluorescence of the reporter dye of the HIV-1 probe rises above the baseline fluorescence level (see Figure 1). Since the amount of HIV-1 QS RNA is constant between all specimens, the fluorescence of the reporter dye of the HIV-1 QS probe should appear at the same cycle for all specimens (see Figure 2). In specimens where the QS fluorescence is affected, the concentration is adjusted accordingly. The appearance of the specific fluorescent signals is reported as a critical threshold value (Ct). The Ct is defined as the fractional cycle number where reporter dye fluorescence exceeds a pre-determined threshold (the Assigned Fluorescence Level), and starts the exponential growth phase of this signal (see Figure 3). A higher Ct value indicates a lower titer of initial HIV-1 target material. A 2-fold increase in titer correlates with a decrease of 1 Ct for target HIV-1 RNA, while a 10-fold increase in titer correlates with a decrease of 3.3 Ct.

Figure 1 shows the target growth curves for a dilution series spanning a 5-$\log_{10}$ range. As the concentration of the virus increases, the growth curves shift to earlier cycles. Therefore, the leftmost growth curve corresponds to the highest viral titer level, whereas, the rightmost growth curve corresponds to the lowest viral titer level.

***Figure 1***
***Target Growth Curves for a Dilution Series Spanning a 5-$\log_{10}$ Range***

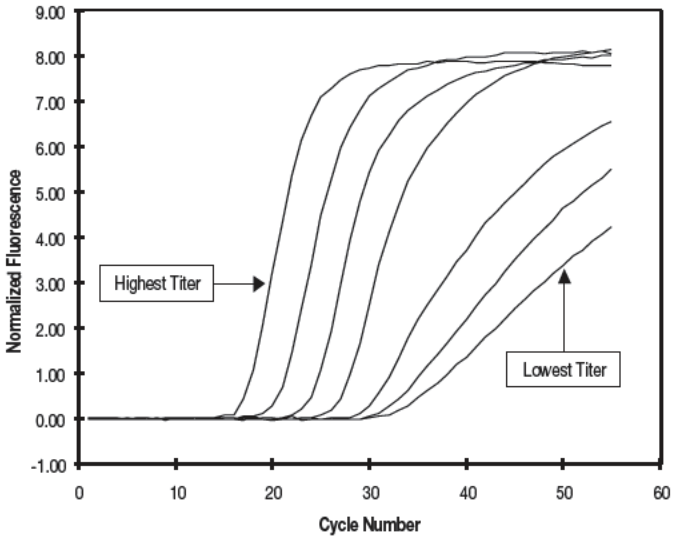



Figure 2 shows the Quantitation Standard growth curves for specimens from a viral dilution series that spans a 5-$\log_{10}$ range. The amount of Quantitation Standard added to each specimen is constant for each reaction. The Ct value of the Quantitation Standard is similar regardless of the target viral titer.

***Figure 2***
***Quantitation Standard Growth Curves for Specimens from a Viral Dilution Series that Spans a 5-$\log_{10}$ Range***

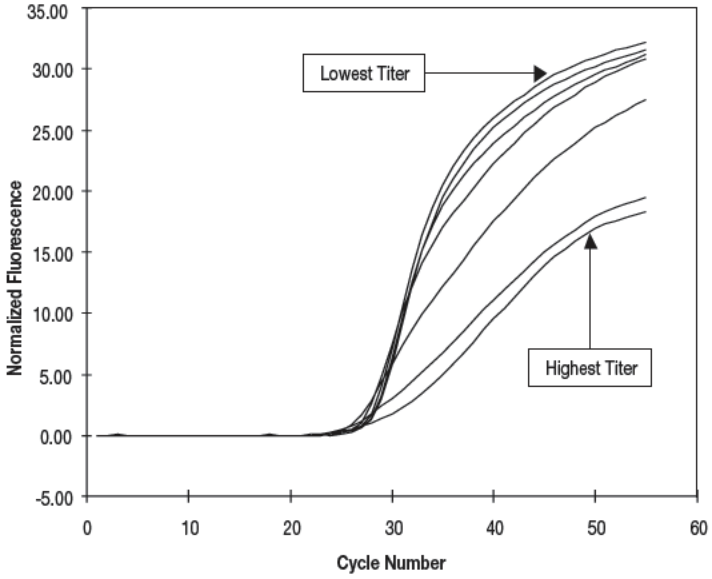
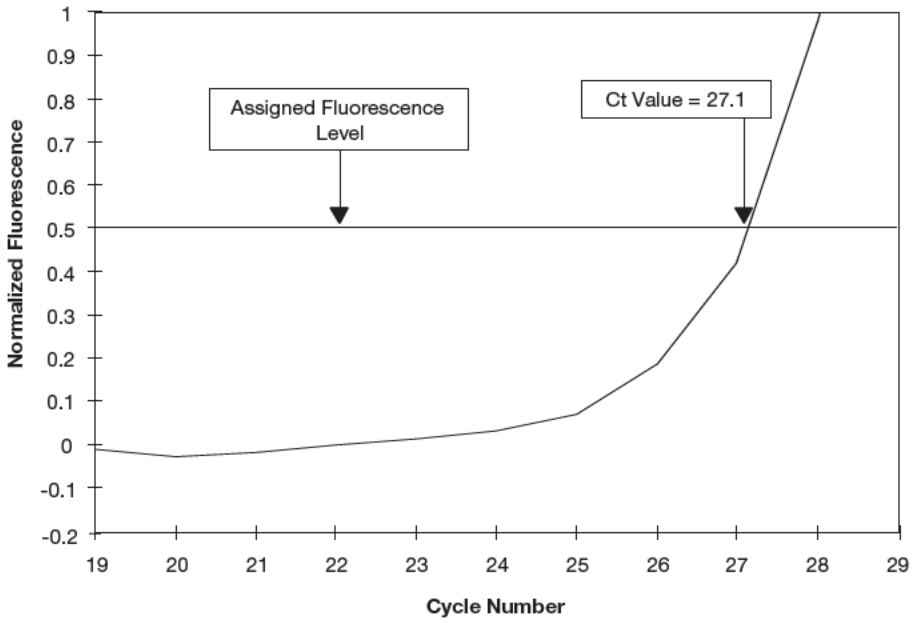


Figure 3 provides an example of how the fluorescence values at every cycle are normalized for each growth curve. The critical threshold value (Ct) is calculated where the fluorescence signal crosses the Assigned Fluorescence Level.

***Figure 3***
***Normalization of Fluorescence Values at Every Cycle for Each Growth Curve***




**HIV-1 RNA Quantitation**

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test quantitates HIV-1 viral RNA by utilizing a second target sequence (HIV-1 Quantitation Standard) that is added to each test specimen at a known concentration. The HIV-1 QS is a non-infectious Armored RNA construct, containing fragments of HIV-1 sequences with primer binding regions identical to those of the HIV-1 target sequence. The HIV-1 QS contains HIV-1 primer binding regions and generates an amplification product of the same length and base composition as the HIV-1 target RNA. The detection probe binding region of the HIV-1 QS has been modified to differentiate HIV-1 QS amplicon from HIV-1 target amplicon.

During the annealing phase of the PCR on the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer, the specimens are illuminated and excited by filtered light and filtered emission fluorescence data are collected for each specimen. The readings from each specimen are then corrected for instrumental fluctuations. These fluorescence readings are sent by the instrument to the AMPLILINK software and stored in a database. Pre-Checks are used to determine if the HIV-1 RNA and HIV-1 QS RNA data represent sets that are valid, and flags are generated when the data lie outside the preset limits. After all Pre-Checks are completed and passed, the fluorescence readings are processed to generate Ct values for the HIV-1 RNA and the HIV-1 QS RNA. The lot-specific calibration constants provided with the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test are used to calculate the titer value for the specimens and controls based upon the HIV-1 RNA and HIV-1 QS RNA Ct values. The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is standardized against the WHO 1st International Standard for HIV-1 RNA for Nucleic Acid-Based Techniques (NAT) (NIBSC 97/656)[36]. Titer results are reported in copies/mL (cp/mL). The conversion factor between reported HIV-1 RNA copies/mL and HIV-1 International Units/mL has been determined by Roche Molecular Systems, Inc. to be 0.6 cp/IU (1.7 IU/cp).

**REAGENTS**

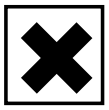
**COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test** HIMCAP 48 Tests

(P/N: 03542998 190)

**HIV-1 CS1** 1 x 48 Tests

(HIV-1 Magnetic Glass Particles Reagent Cassette) 1 x 7.0 mL

Magnetic glass particles
93% Isopropanol

Xi  93% (w/w) Isopropanol

Irritant

F 93% (w/w) Isopropanol

Highly Flammable

**HIV-1 CS2** 1 x 48 Tests

(HIV-1 Lysis Reagent Cassette) 1 x 78 mL

Sodium citrate dihydrate
42.5% Guanidine thiocyanate
< 14% Polydocanol
0.9% Dithiothreitol

Xn 42.5% (w/w) Guanidine thiocyanate

Harmful

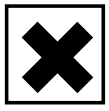
**HIV-1 CS3** 1 x 48 Tests

HIV-1 Multi-Reagent Cassette containing:

**Pase** 1 x 3.8 mL

(Proteinase Solution)

Tris buffer
< 0.05% EDTA
Calcium chloride
Calcium acetate
≤ 7.8% Proteinase
Glycerol

Xn  ≤ 7.8% (w/w) Proteinase

Harmful

**EB** 1 x 7.0 mL

(Elution Buffer)

Tris-base buffer
0.2% Methylparaben

**HIV-1 CS4** 1 x 48 Tests

HIV-1 Test-Specific Reagent Cassette containing:

**HIV-1 QS** 1 x 3.6 mL

(HIV-1 Quantitation Standard)

Tris-HCl buffer
EDTA
< 0.005% Poly rA RNA (synthetic)
< 0.001% Armored HIV-1 RNA construct containing HIV-1 primer binding sequences and a unique probe binding region (non-infectious RNA in MS2 bacteriophage)
0.05% Sodium azide

**HIV-1 MMX** 1 x 2.5 mL
(HIV-1 Master Mix)

Tricine buffer
Potassium acetate
Potassium hydroxide
20% Dimethylsulfoxide
Glycerol
< 0.04% dATP, dCTP, dGTP, dUTP, dTTP
< 0.003% Upstream and downstream primers to the GAG region of HIV-1
< 0.003% Oligonucleotide aptamer
< 0.003% Fluorescent-labeled oligonucleotide probes specific for HIV-1 and the HIV-1 QS
< 0.05% Z05 DNA Polymerase (microbial)
< 0.1% AmpErase (uracil-N-glycosylase) enzyme (microbial)
0.09% Sodium azide

**CAP/CTM $Mn^{2+}$** 1 x 19.8 mL
(CAP/CTM Manganese Solution)

< 0.5% Manganese acetate
Glacial acetic acid
0.09% Sodium azide

**HIV-1 H(+)C** 4 x 1.0 mL
(HIV-1 High Positive Control)

< 0.001% Armored HIV-1 RNA construct containing HIV-1 sequences (non-infectious RNA in MS2 bacteriophage)
Negative Human Plasma, non-reactive by FDA licensed tests for antibody to HCV, antibody to HIV-1/2, HIV p24 antigen and HBsAg; HIV-1 RNA, HCV RNA and HBV DNA not detectable by PCR methods
0.1% ProClin® 300 preservative

**HIV-1 L(+)C** 4 x 1.0 mL
(HIV-1 Low Positive Control)

< 0.001% Armored HIV-1 RNA construct containing HIV-1 sequences (non-infectious RNA in MS2 bacteriophage) at a mean concentration at least 100 fold lower than the mean concentration of Armored HIV-1 RNA in HIV-1 H(+)C
Negative Human Plasma, non-reactive by FDA licensed tests for antibody to HCV, antibody to HIV-1/2, HIV p24 antigen and HBsAg; HIV-1 RNA, HCV RNA and HBV DNA not detectable by PCR methods
0.1% ProClin® 300 preservative

**CTM (–) C** 4 x 1.0 mL
[COBAS® TaqMan® Negative Control (Human Plasma)]

Negative Human Plasma, non-reactive by FDA licensed tests for antibody to HCV, antibody to HIV-1/2, HIV p24 antigen and HBsAg; HIV-1 RNA, HCV RNA and HBV DNA not detectable by PCR methods
0.1% ProClin® 300 preservative

**HIV-1 H(+)C Clip** 1 x 4 Clips
(HIV-1 High Positive Control Barcode Clip)

**HIV-1 L(+)C Clip** 1 x 4 Clips
(HIV-1 Low Positive Control Barcode Clip)

**HIV-1 (–) C Clip** 1 x 4 Clips
(HIV-1 Negative Control Barcode Clip)

PG WR

**COBAS® AmpliPrep/COBAS® TaqMan® Wash Reagent**
(P/N: 03587797 190)

**PG WR** 1 x 5.1 L
(COBAS® AmpliPrep/COBAS® TaqMan® Wash Reagent)

Sodium citrate dihydrate
< 0.1% N-Methylisothiazolone-HCl

## WARNINGS AND PRECAUTIONS

A. **FOR *IN VITRO* DIAGNOSTIC USE.**

B. This test is for use with human plasma collected in the anticoagulant EDTA.

C. Do not pipet by mouth.

D. Do not eat, drink or smoke in laboratory work areas. Wear protective disposable gloves, laboratory coats and eye protection when handling specimens and kit reagents. Wash hands thoroughly after handling specimens and test reagents.

E. Avoid microbial and ribonuclease contamination of reagents when removing aliquots from control vials.

F. The use of sterile disposable pipets and RNase-free pipet tips is recommended.

G. Do not pool controls from different lots or from different vials of the same lot.

H. Do not mix reagent cassettes or controls from different kits.

I. Do not allow reagent cassettes to come to ambient temperature outside the COBAS® AmpliPrep Instrument.

J. Do not open COBAS® AmpliPrep cassettes and exchange, mix, remove or add bottles.

K. Dispose of unused reagents, waste and specimens in accordance with country, federal, state and local regulations.

L. Do not use a kit after its expiration date.

M. Material Safety Data Sheets (MSDS) are available on request from your local Roche office.

N. Specimens and controls should be handled as if infectious using safe laboratory procedures such as those outlined in *Biosafety in Microbiological and Biomedical Laboratories*[37] and in the CLSI Document M29-A[38]. Thoroughly clean and disinfect all work surfaces with a freshly prepared solution of 0.5% sodium hypochlorite in deionized or distilled water.

***NOTE: Commercial liquid household bleach typically contains sodium hypochlorite at a concentration of 5.25%. A 1:10 dilution of household bleach will produce a 0.5% sodium hypochlorite solution. Do not autoclave bleach solution.***

O. **CAUTION: CTM (–) C, HIV-1 L(+)C** and **HIV-1 H(+)C** contain Human Plasma derived from human blood. The source material has been tested by FDA licensed tests and found non-reactive for the presence of Hepatitis B Surface Antigen (HBsAg), antibodies to HIV-1/2 and HCV, and HIV p24 Antigen. Testing of Negative Human Plasma by PCR methods showed no detectable HIV-1 RNA, HCV RNA or HBV DNA. No known test methods can offer complete assurance that products derived from human blood will not transmit infectious agents. Therefore, all human sourced material should be considered potentially infectious. **CTM (–) C, HIV-1 L(+)C** and **HIV-1 H(+)C** should be handled as if infectious using safe laboratory procedures such as those outlined in *Biosafety in Microbiological and Biomedical Laboratories*[37] and in the CLSI Document M29-A[38]. Thoroughly clean and disinfect all work surfaces (for instrument work surfaces see COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer and the AMPLILINK Software, Version 3.1.x Series) with a freshly prepared solution of 0.5% sodium hypochlorite in deionized or distilled water.

P. **HIV-1 QS, CAP/CTM $Mn^{2+}$** and **HIV-1 MMX** contain sodium azide. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. While disposing of sodium azide-containing solutions down laboratory sinks, flush the drains with a large volume of water to prevent azide buildup.

Q. Wear eye protection, laboratory coats and disposable gloves when handling any reagent. Avoid contact of these materials with the skin, eyes or mucous membranes. If contact does occur, immediately wash with large amounts of water. Burns can occur if left untreated. If spills of these reagents occur, dilute with water before wiping dry.

R. Do not allow **HIV-1 CS2** and liquid waste from the COBAS® AmpliPrep Instrument, which contain guanidine thiocyanate, to contact sodium hypochlorite (bleach) solution. These mixtures can produce a highly toxic gas.

S. When disposing of used COBAS® AmpliPrep Sample Processing Units (SPUs), which contain guanidine thiocyanate, avoid any contact with sodium hypochlorite (bleach) solution. These mixtures can produce a highly toxic gas.

## STORAGE AND HANDLING REQUIREMENTS

A. ***Do not freeze reagents or controls.***

B. Store **HIV-1 CS1, HIV-1 CS2, HIV-1 CS3** and **HIV-1 CS4** at 2-8ºC. Unused, these reagents are stable until the expiration date indicated. Once used, these reagents are stable for 28 days at 2-8°C or until the expiration date, whichever comes first. **HIV-1 CS1, HIV-1 CS2, HIV-1 CS3** and **HIV-1 CS4** can be used for a maximum of 4 instrument cycles, up to a maximum of 64 hours cumulative on board the COBAS® AmpliPrep Instrument. Reagents must be stored at 2-8°C between instrument cycles.

C. Store **HIV-1 H(+)C, HIV-1 L(+)C** and **CTM (–) C** at 2-8ºC. The controls are stable until the expiration date indicated. Once opened, any unused portion must be discarded.

D. Store Barcode clips [**HIV-1 H(+)C Clip, HIV-1 L(+)C Clip** and **HIV-1 (–) C Clip**] at 2-30°C.

E. Store **PG WR** at 2-30°C. **PG WR** is stable until the expiration date indicated. Once opened, this reagent is stable for 28 days at 2-30°C or until the expiration date, whichever comes first.

## MATERIALS PROVIDED

A. **COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test** HIMCAP
(P/N: 03542998 190)

**HIV-1 CS1**
(HIV-1 Magnetic Glass Particles Reagent Cassette)

**HIV-1 CS2**
(HIV-1 Lysis Reagent Cassette)

**HIV-1 CS3**
(HIV-1 Multi-Reagent Cassette)

**HIV-1 CS4**
(HIV-1 Test-Specific Reagent Cassette)

**HIV-1 H(+)C**
(HIV-1 High Positive Control)

**HIV-1 L(+)C**
(HIV-1 Low Positive Control)

**CTM (–) C**
[COBAS® TaqMan® Negative Control (Human Plasma)]

**HIV-1 H(+)C Clip**
(HIV-1 High Positive Control Barcode Clip)

**HIV-1 L(+)C Clip**
(HIV-1 Low Positive Control Barcode Clip)

**HIV-1 (–) C Clip**
(HIV-1 Negative Control Barcode Clip)

B. **COBAS® AmpliPrep/COBAS® TaqMan® Wash Reagent** PG WR
(P/N: 03587797 190)

**PG WR**
(COBAS® AmpliPrep/COBAS® TaqMan® Wash Reagent)

## MATERIALS REQUIRED BUT NOT PROVIDED

### Instrumentation and Software

- COBAS® AmpliPrep Instrument
- COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer
- Optional: Docking Station
- AMPLILINK Software, Version 3.1.x Series
- Data Station for the AMPLILINK software, with printer
- COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer and the AMPLILINK Software, Version 3.1.x Series
- COBAS® TaqMan® Analyzer (plus optional Docking Station) Instrument Manual for use with AMPLILINK Software, Version 3.1.x Series
- COBAS® TaqMan® 48 Analyzer Instrument Manual for use with AMPLILINK Software, Version 3.1.x Series
- AMPLILINK Software Version 3.1.x Series Application Manual for use with the COBAS® AmpliPrep Instrument, COBAS® TaqMan® Analyzer, and COBAS® TaqMan® 48 Analyzer

- COBAS® TaqMan® Analyzer HIV-1 PCR TEST FILE CD ROM
- COBAS® TaqMan® 48 Analyzer HIV-1 PCR TEST FILE CD ROM

**Disposables**

- Sample processing units: SPUs (P/N: 03755525001)
- Sample input tubes (S-tubes) with barcode clips (P/N: 03137040001)
- Racks of K-tips (P/N: 03287343001)
- K-tube Box of 12 x 96 (P/N: 03137082001)

**OTHER MATERIALS REQUIRED BUT NOT PROVIDED**

- Sample Rack (SK 24 rack) (P/N: 28122172001)
- Reagent Rack (P/N: 28122199001)
- SPU rack (P/N: 28122806001)
- K-tube capper, motorized (P/N: 03516539001)
- K-tube capper (P/N: 03339874001)
- K-carrier (P/N: 28150397001)
- K-carrier Transporter (P/N: 03517519001)
- K-carrier rack (P/N: 03286436001)
- Pipettors with aerosol barrier or positive displacement RNase-free tips (capacity 1000 µL)*
- Disposable gloves, powderless
- Vortex mixer

* Pipettors should be accurate within 3% of stated volume. Aerosol barrier or positive displacement RNase-free tips must be used where specified to prevent specimen and amplicon cross-contamination.

**SPECIMEN COLLECTION, TRANSPORT AND STORAGE**

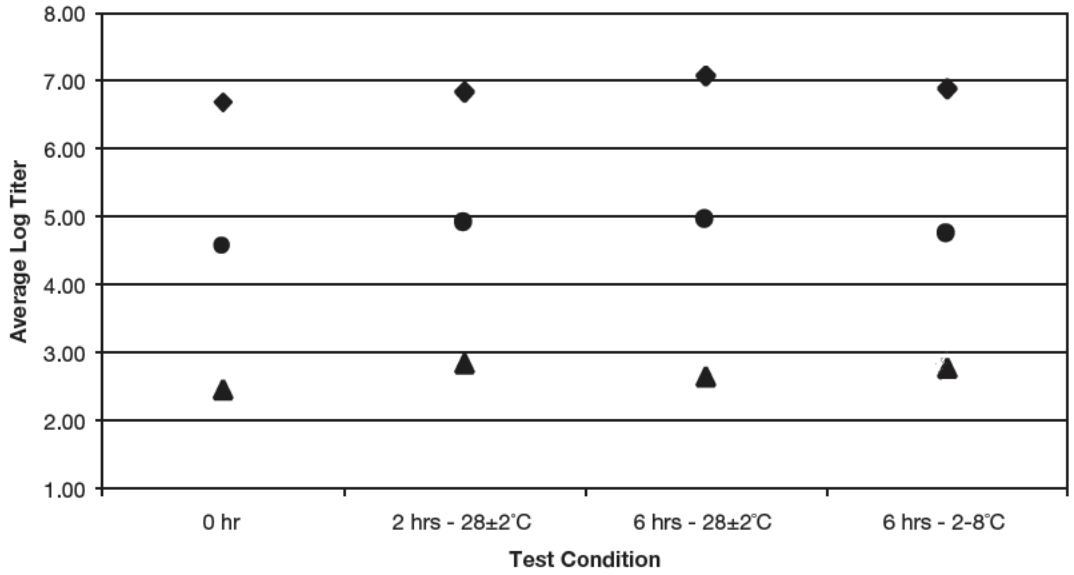
***NOTE: Handle all specimens and controls as if they are capable of transmitting infectious agents.***

**A. Specimen Collection**

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is for use with plasma specimens. Blood should be collected in sterile tubes using EDTA (lavender top) as the anticoagulant.

Store whole blood at 2-25°C for no longer than 6 hours. Separate plasma from whole blood within 6 hours of collection by centrifugation at 800-1600 x g for 20 minutes at room temperature. Transfer plasma to a sterile polypropylene tube. Figures 4 and 5 show the data from specimen collection studies. These studies were performed using the COBAS® TaqMan® HIV-1 Test for use with the High Pure System (HPS), P/N: 03501752 190 and 03502295 001).

***Figure 4***
***HIV-1 Stability in Whole Blood with EDTA Anticoagulant***



## B. Specimen Transport

Transportation of whole blood or plasma must comply with country, federal, state and local regulations for the transport of etiologic agents[39]. Whole blood must be transported at 2-25°C and centrifuged within 6 hours of collection. Plasma may be stored at room temperature for up to 1 day, at 2-8°C for up to 5 days or frozen at -20°C to -80°C for longer storage. Figure 5 shows the stability for HIV-1 in EDTA plasma.

## C. Specimen Storage

Plasma specimens may be stored at room temperature for up to 1 day, at 2-8°C for up to 5 days or frozen at -20°C to -80°C. It is recommended that specimens be stored in 1100-1200 µL aliquots in sterile, 2.0 mL polypropylene screw-cap tubes (such as Sarstedt 72.694.006). Plasma specimens may be frozen and thawed up to five times without a loss of HIV-1 RNA. Figure 6 shows the data from a freeze/thaw study (study was performed using COBAS® TaqMan® HIV-1 Test for use with the High Pure System (HPS), P/N: 03501752 190 and 03502295 001).

***Figure 5***
***HIV-1 Stability in EDTA-Plasma***

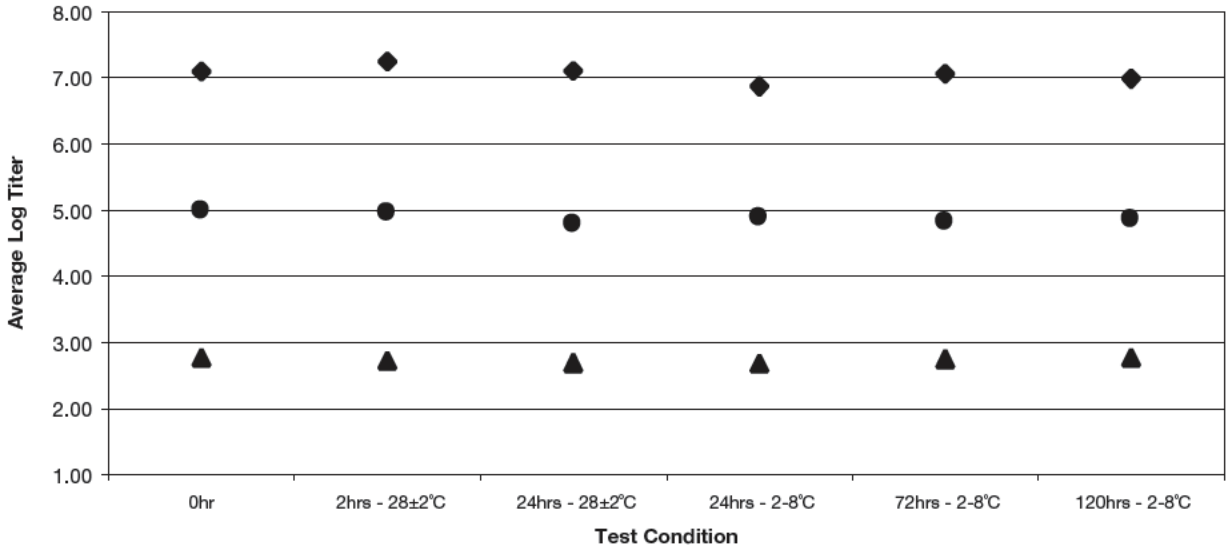
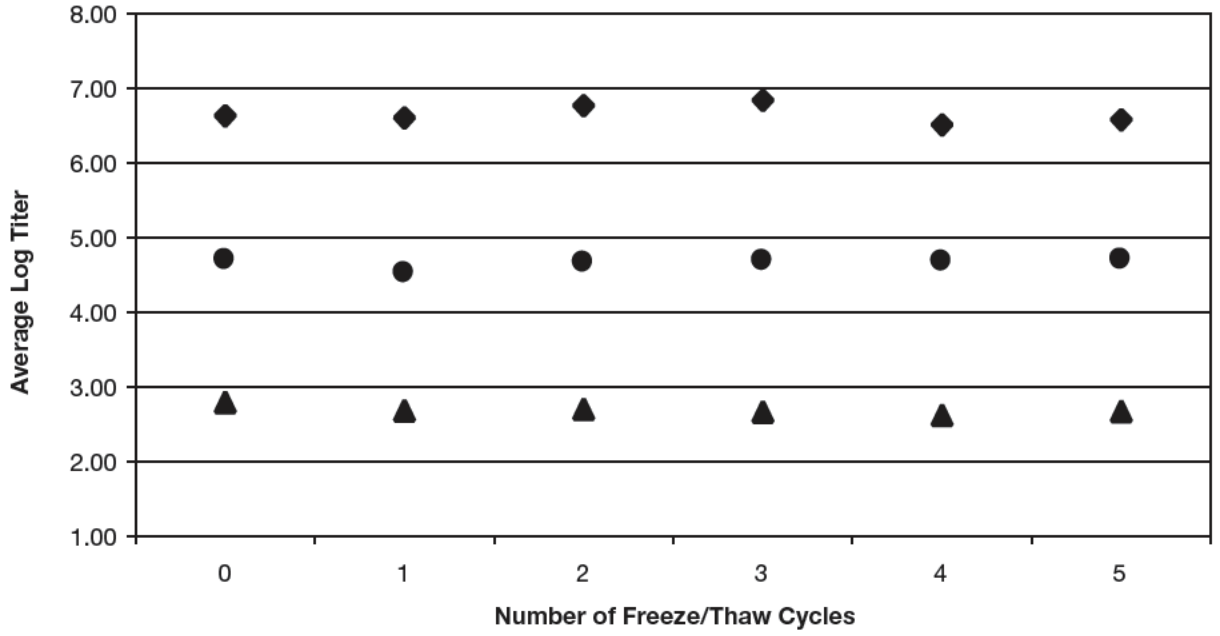



***Figure 6***
***HIV-1 Results after up to Five Freeze/Thaw Cycles***



## INSTRUCTIONS FOR USE

***NOTE: Refer to the following for detailed operating instructions: (a) the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer and the AMPLILINK Software, Version 3.1.x Series; and either (b) the COBAS® TaqMan® Analyzer (plus optional Docking Station) Instrument Manual for use with AMPLILINK Software, Version 3.1.x Series or (c) the COBAS® TaqMan® 48 Analyzer Instrument Manual for use with AMPLILINK Software, Version 3.1.x Series.***

**Batch Size:**

Each kit contains reagents sufficient for 48 tests, which may be performed in batches of 12 to 24 tests. At least one replicate each of **CTM (–) C**, **HIV-1 L(+)C** and **HIV-1 H(+)C** must be included in each batch (see "*Quality Control*" section).

**Workflow:**

The COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer run must be started within 120 minutes following completion of specimen and control preparation of a specific batch.

***NOTE: If this time limit is exceeded, processed specimens and controls must be discarded.***

***(Processing time for one specimen on the COBAS® AmpliPrep Instrument is 216 seconds. The COBAS® AmpliPrep Instrument can process three racks of 24 specimens (n = 72) in approximately 5 hours. The amplification and detection cycle takes 3 hours, 5 minutes on both the COBAS® TaqMan® Analyzer and COBAS® TaqMan® 48 Analyzer. Completion of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test takes approximately 8 hours 10 minutes and 7 hours 35 minutes for sample processing and result generation, using the CTM 48 and CTM 96 workflows, respectively).***

***NOTE: DO NOT FREEZE or STORE processed specimens and controls at 2-8°C.***

## Specimen and Control Preparation

***NOTE: If using frozen specimens, place the specimens at room temperature until completely thawed and vortex for 3-5 seconds before use. Controls should be removed from 2-8°C storage and equilibrated to ambient temperature before use.***

### *COBAS® AmpliPrep Instrument Set-up*

**Part A. Maintenance and Priming**

A1. The COBAS® AmpliPrep Instrument is ready for operation in stand-by mode.

A2. Turn the Data Station for the AMPLILINK software **ON**. Prepare the Data Station as follows:

a. Log onto Windows® XP.

b. Double click the AMPLILINK software icon.

c. Log onto AMPLILINK software by entering the assigned User ID and password.

A3. Check the supply of **PG WR** using the **Status** Screen and replace if necessary.

A4. Perform all Maintenance that is listed in the Due Tab, as outlined in the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer and the AMPLILINK Software, Version 3.1.x Series. The COBAS® AmpliPrep Instrument will automatically prime the system.

**Part B. Loading of Reagent Cassettes**

***NOTE: All reagent cassettes should be removed from 2-8°C storage, immediately loaded onto the COBAS® AmpliPrep Instrument and allowed to equilibrate to ambient temperature on the instrument for at least 30 minutes before the first specimen is to be processed. Do not let reagent cassettes come to ambient temperature outside the instrument.***

B1. Place **HIV-1 CS1** onto a reagent rack. Place **HIV-1 CS2, HIV-1 CS3** and **HIV-1 CS4** onto a separate reagent rack.

B2. Load the reagent rack containing **HIV-1 CS1** onto rack position **A** of the COBAS® AmpliPrep Instrument as described in the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer and the AMPLILINK Software, Version 3.1.x Series.

B3. Load the reagent rack containing **HIV-1 CS2, HIV-1 CS3** and **HIV-1 CS4** onto rack position **B, C, D** or **E** of the COBAS® AmpliPrep Instrument as described in the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer and the AMPLILINK Software, Version 3.1.x Series (see also Table 1 for additional information).

**Part C. Loading of Disposables**

***NOTE: Determine the number of COBAS® AmpliPrep reagent cassettes, Sample Processing Units (SPUs), Input Sample tubes (S-tubes), K-tips and K-tubes needed. One SPU, one Input S-tube, one K-tip and one K-tube are needed for each specimen or control.***

Multiple configurations for use of the COBAS® AmpliPrep Instrument with the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer are possible. For reference, see Table 1 below. Depending on the configuration used, load the appropriate number of reagent cassette racks, sample racks with Input S-tubes, SPU racks, K-tip racks, K-tube racks and K-carriers on K-carrier racks onto the respective rack positions of the COBAS® AmpliPrep Instrument as described in the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer and the AMPLILINK Software, Version 3.1.x Series (*also see* Table 1 for additional information).

C1. Place the SPUs in the SPU rack(s) and load the rack(s) onto rack position **J, K** or **L** of the COBAS® AmpliPrep Instrument as described in the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer and the AMPLILINK Software, Version 3.1.x Series.

C2. Depending on the configuration used, load full K-tube rack(s) onto rack position **M, N, O** or **P** of the COBAS® AmpliPrep Instrument as described in the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer and the AMPLILINK Software, Version 3.1.x Series.

C3. Load full K-tip rack(s) onto rack position **M, N, O** or **P** of the COBAS® AmpliPrep Instrument as described in the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer and the AMPLILINK Software, Version 3.1.x Series.

C4. For configurations 3 to 5 using the COBAS® TaqMan® 48 Analyzer, load K-carriers on K-carrier rack(s) onto rack position **M, N, O** or **P** of the COBAS® AmpliPrep Instrument as described in the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer and the AMPLILINK Software, Version 3.1.x Series.

***Table 1***
***Possible Configurations for using COBAS® AmpliPrep Instrument with COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer***

| Configuration | | Transfer mode to COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer | Racks, carriers and disposables | Position on COBAS® AmpliPrep Instrument |
|---|---|---|---|---|
| **1.** | COBAS® AmpliPrep Instrument plus Docking Station plus COBAS® TaqMan® Analyzer | Automated transfer of K-carrier | K-tubes in full K-tube racks<br>K-tips in full K-tips racks<br>Input S-tubes containing specimens and controls on sample racks<br>SPUs in SPU racks<br>CS1 on Cassette rack<br>CS2, CS3, CS4 on Cassette rack | M-P<br>M-P<br>F-H<br><br><br>J-L<br>A<br>B-E |
| **2** | COBAS® AmpliPrep Instrument plus COBAS® TaqMan® Analyzer | Manual transfer of K-tubes via sample rack(s) onto COBAS® TaqMan® Analyzer | K-tubes in full K-tube racks<br>K-tips in full K-tips racks<br>Input S-tubes on sample racks<br>SPUs in SPU racks<br>CS1 on Cassette rack<br>CS2, CS3, CS4 on Cassette rack<br><u>After specimen processing is finished:</u><br>K-tubes on sample racks (ready for manual transfer) | M-P<br>M-P<br>F-H<br>J-L<br>A<br>B-E<br><br><br>Same as above (F-H) |
| **3. & 4.** | COBAS® AmpliPrep Instrument plus 1-2 COBAS® TaqMan® 48 Analyzer(s) | Manual transfer of K-carrier via K-carrier rack(s) onto COBAS® TaqMan® 48 Analyzer | K-tubes on sample racks<br>K-tips in full K-tips racks<br>Input S-tubes on sample racks<br>SPUs in SPU racks<br>CS1 on Cassette rack<br>CS2, CS3, CS4 on Cassette rack<br>Empty barcoded K-carrier on K-carrier rack<br><u>After specimen processing is finished:</u><br>K-tubes in K-carrier on K-carrier rack | F-H<br>M-P<br>F-H<br>J-L<br>A<br>B-E<br>M-P<br><br><br>Same as above (M-P) |
| **5.** | COBAS® AmpliPrep Instrument plus COBAS® TaqMan® Analyzer plus COBAS® TaqMan® 48 Analyzer | Manual transfer of K-tubes on sample rack to COBAS® TaqMan® Analyzer. Manual transfer of K-carrier via K-carrier rack to COBAS® TaqMan® 48 Analyzer | Same as configurations 2 and 3 | Same as configurations 2 and 3 |

**Part D. Ordering and Loading of Specimens**

D1. Prepare sample racks as follows: Attach a barcode label clip to each sample rack position where a specimen (S-tube) is to be placed. Attach one of the specific barcode label clips for the controls [**CTM (–) C, HIV-1 L(+)C and HIV-1 H(+)C**] to each sample rack position where the controls (S-tube) are to be placed. The barcode label clips for controls must have the same control lot number as the lot number on the control vials in the kit. Take care in assigning the correct control to the position with the appropriate control barcode clip. Place one Input S-tube into each position containing a barcode label clip.

D2. Using the AMPLILINK software, create specimen orders for each specimen and control in the **Orders** window **Sample** folder. The Test file name in the **Orders** window will be HIMAPU96 or HIMAPU48. Select the appropriate test file and complete by saving.

D3. Assign specimen and control orders to sample rack positions in the **Orders** window **Sample Rack** folder. The sample rack number must be entered for the rack prepared in Step D1.

D4. Print the **Sample Rack Order** report to use as a worksheet.

D5. Prepare specimen and control racks in the designated area for specimen and control addition as follows: Vortex each specimen and control [**CTM (–) C, HIV-1 L(+)C** and **HIV-1 H(+)C**] for 3 to 5 seconds. Avoid contaminating gloves when manipulating the specimens and controls.

D6. Transfer 1000 to 1050 µL of each specimen and control [**CTM (–) C, HIV-1 L(+)C** and **HIV-1 H(+)C**] to the appropriate barcode labeled Input S-tube using a micropipettor with an aerosol barrier or positive displacement RNase-free tip. ***Avoid transferring particulates and/or fibrin clots from the original specimen to the Input S-tube.*** Specimens and controls should be transferred to tube positions as assigned and recorded on the worksheet in step D4. The barcode label clips for controls must have the same control lot number as the lot number on the control vials in the kit. Assign the correct control to the position with the appropriate control barcode clip. ***Avoid contaminating the upper part of the S-tubes with specimens or controls.***

D7. For configurations 1 and 2 using the COBAS® TaqMan® Analyzer, load the sample rack(s) filled with Input S-tubes onto rack positions **F, G** or **H** of the COBAS® AmpliPrep Instrument.

D8. For configurations 3 to 5 using the COBAS® TaqMan® 48 Analyzer, load sample rack(s) with Input S-tubes and K-tubes (one for each Input S-tube, loaded in the correct position adjacent to Input S-tubes) onto rack position **F, G** or **H** of the COBAS® AmpliPrep Instrument as described in the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer and the AMPLILINK Software, Version 3.1.x Series.

**Part E. Start of COBAS® AmpliPrep Instrument Run**

E1. Start the COBAS® AmpliPrep Instrument using the AMPLILINK software as described in the AMPLILINK Software Version 3.1.x Series Application Manual for use with the COBAS® AmpliPrep Instrument, COBAS® TaqMan® Analyzer, and COBAS® TaqMan® 48 Analyzer.

**Part F. End of COBAS® AmpliPrep Instrument Run and Transfer to COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer (only for configurations 2-5)**

F1. Check for flags or error messages in the system screen as described in (a) the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer and the AMPLILINK Software, Version 3.1.x Series and (b) the AMPLILINK Software Version 3.1 Series Application Manual for use with the COBAS® AmpliPrep Instrument, COBAS® TaqMan® Analyzer, and COBAS® TaqMan® 48 Analyzer.

F2. Remove processed specimens and controls from the COBAS® AmpliPrep Instrument on either sample racks (for COBAS® TaqMan® Analyzer without docking station) or K-carrier racks (for COBAS® TaqMan® 48 Analyzer), depending on the configuration (for further details see Part G).

F3. Remove waste from COBAS® AmpliPrep Instrument.

***NOTE: All processed specimens and controls should not be exposed to light after completion of specimen and control preparation.***

## Amplification and Detection

### *COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer Set-up*

The COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer run must be started within 120 minutes following completion of specimen and control preparation. If this time limit is exceeded, the processed specimens and controls must be discarded.

***NOTE: DO NOT FREEZE or STORE processed specimens and controls at 2-8°C.***

For a detailed description of the possible configurations, refer to (a) the COBAS® TaqMan® Analyzer (plus optional Docking Station) Instrument Manual for use with AMPLILINK Software, Version 3.1.x Series; (b) the COBAS® TaqMan® 48 Analyzer Instrument Manual for use with AMPLILINK Software, Version 3.1.x Series and Part C of this Package Insert.

**Part G. Loading Processed Specimens**

G1. Depending on the instrument configuration, perform the appropriate steps to transfer the K-tubes to the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer:

*Configuration 1:* Automated transfer of K-carrier via docking station to COBAS® TaqMan® Analyzer. Manual intervention is unnecessary.

*Configuration 2 and 5:* Manual transfer of K-tubes in sample rack(s) to COBAS® TaqMan® Analyzer

*Configuration 3, 4 and 5:* Manual transfer of K-carrier on K-carrier rack(s) to the COBAS® TaqMan® 48 Analyzer. Manual transfer of K-carriers into COBAS® TaqMan® 48 Analyzer using the K-carrier Transporter.

**Part H. Start of COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer Run**

H1. Start the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer by one of the options below depending on the instrument configuration used:

*Configuration 1:* No intervention necessary.

*Configuration 2 and 5:* Automatic start of the COBAS® TaqMan® Analyzer after insertion of sample rack(s).

*Configuration 3, 4 and 5:* Fill K-carrier with empty K-tubes if there are fewer than 6 K-tubes on the K-carrier. Filling is guided by the AMPLILINK software. Open thermal cycler cover, load K-carrier into thermal cycler and close lid. Start the COBAS® TaqMan® 48 Analyzer run.

**Part I. End of COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer Run**

I1. At the completion of the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer run, print Results Report. Check for flags or error messages in the Result Report as described in (a) the COBAS® TaqMan® Analyzer (plus optional Docking Station) Instrument Manual for use with AMPLILINK Software, Version 3.1.x Series or (b) the COBAS® TaqMan® 48 Analyzer Instrument Manual for use with AMPLILINK Software, Version 3.1.x Series and (c) the AMPLILINK Software Version 3.1.x Series Application Manual for use with the COBAS® AmpliPrep Instrument, COBAS® TaqMan® Analyzer, and COBAS® TaqMan® 48 Analyzer. Specimens with flags and comments are interpreted as described in the *Results* section. After acceptance, store data in archive.

I2. Remove used K-tubes from the COBAS® TaqMan® 48 Analyzer. If using the COBAS® TaqMan® 48 Analyzer, tubes are transferred to a waste container, which should be emptied as described in the COBAS® TaqMan® Analyzer Instrument Manual.

***NOTE: Caution! K-carrier may be hot after cycling.***

## RESULTS

The COBAS® TaqMan® Analyzer or the COBAS® TaqMan® 48 Analyzer automatically determines the HIV-1 RNA concentration for the specimens and controls. **The HIV-1 RNA concentration is expressed in copies (cp)/mL. The conversion factor between HIV-1 RNA copies/mL and HIV-1 International Units (IU)/mL is 0.6 cp/IU, using the WHO 1st International Standard for HIV-1 RNA for Nucleic Acid-Based Techniques (NAT) (NIBSC 97/656).**

**AMPLILINK Software:**

- Determines the Cycle Threshold value (Ct) for the HIV-1 RNA and the HIV-1 QS RNA.
- Determines the HIV-1 RNA concentration based upon the Ct values for the HIV-1 RNA and HIV-1 QS RNA and the lot-specific calibration coefficients provided on the cassette barcodes.
- Determines that the calculated cp/mL for **HIV-1 L(+)C** and **HIV-1 H(+)C** fall within the assigned ranges.

### Batch Validation

Check AMPLILINK software results window or printout for flags and comments to ensure that the batch is valid.

***NOTE: For instructions on printing results and for interpreting flags and comments, please refer to the following: (a) the AMPLILINK Software Version 3.1.x Series Application Manual for use with the COBAS® AmpliPrep Instrument, COBAS® TaqMan® Analyzer, and COBAS® TaqMan® 48 Analyzer or (b) the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer and the AMPLILINK Software, Version 3.1.x Series and (c) the COBAS® TaqMan® Analyzer (plus optional Docking Station) Instrument Manual for use with AMPLILINK Software, Version 3.1.x Series or (d) the COBAS® TaqMan® 48 Analyzer Instrument Manual for use with AMPLILINK Software, Version 3.1.x Series.***

For control orders, a check is made to determine if the cp/mL value for the control is within its specified range. If the cp/mL value for the control lies outside of its range, a FLAG is generated to show the control has failed.

The batch is valid if no flags appear for any of the controls [**HIV-1 H(+)C, HIV-1 L(+)C, CTM (–) C**]. The following results are obtained for a valid batch:

| Control | Result | Interpretation |
|---|---|---|
| Negative Control | Target Not Detected | Control within range |
| Low Positive Control | A numeric titer, X.XXE+XX cp/mL | Control within range |
| High Positive Control | A numeric titer, X.XXE+XX cp/mL | Control within range |

The batch is <u>not</u> valid if any of the following flags appear for the HIV-1 Controls:

**Negative Control:**

| Flag | Result | Interpretation |
|---|---|---|
| __N_NC_INVALID | Invalid | An invalid result or a "valid" result that was not negative for HIV-1 target |

**HIV-1 Low Positive Control:**

| Flag | Result | Interpretation |
|---|---|---|
| __L_LPCINVALID | < 4.80E+01 cp/mL | Control below range |
| __L_LPCINVALID | Target Not Detected | Control below range |
| __L_LPCINVALID | A numeric titer, X.XXE+XX cp/mL | Control out of range |
| __L_LPCINVALID | > 1.00E+07 cp/mL | Control above range |
| __L_LPCINVALID | Invalid | An invalid result |

**HIV-1 High Positive Control:**

| Flag | Result | Interpretation |
|---|---|---|
| __H_HPCINVALID | < 4.80E+01 cp/mL | Control below range |
| __H_HPCINVALID | Target Not Detected | Control below range |
| __H_HPCINVALID | A numeric titer, X.XXE+XX cp/mL | Control out of range |
| __H_HPCINVALID | > 1.00E+07 cp/mL | Control above range |
| __H_HPCINVALID | Invalid | An invalid result |

If the batch is invalid, repeat the entire batch including specimen and control preparation, amplification and detection.

**Interpretation of Results:**

For a valid batch, check each individual specimen for flags or comments on the result printout. Interpret the results as follows:

⇒ A valid batch may include both valid and invalid specimen results depending on whether flags and/or comments are obtained for the individual specimens.

**Specimen results are interpreted as follows:**

| Titer Result | Interpretation |
|---|---|
| Target Not Detected | Ct value for HIV-1 above the limit for the assay or no Ct value for HIV-1 obtained. Report results as "HIV-1 RNA not detected". |
| < 4.80E+01 cp/mL | Calculated cp/mL are below the Limit of Detection of the assay. Report results as "HIV-1 RNA detected, less than 48 HIV-1 RNA cp/mL". |
| ≥ 4.80E+01 cp/mL and ≤ 1.00E+07 cp/mL | Calculated results greater than or equal to 48 cp/mL and less than or equal to 1.00E+07 cp/mL are within the Linear Range of the assay. |
| > 1.00E+07 cp/mL | Calculated cp/mL are above the range of the assay. Report results as "greater than 1.00E+07 HIV-1 RNA cp/mL". If quantitative results are desired, the original specimen should be diluted with HIV-1-negative human EDTA-plasma and the test repeated. Multiply the reported result by the dilution factor. |

***NOTE: Specimens above the range of the assay may also produce an Invalid result with a flag "QS_INVALID". If quantitative results are desired, the original specimen should be diluted with HIV-1-negative human EDTA-plasma and the test repeated. Multiply the reported result by the dilution factor.***

## QUALITY CONTROL

One replicate each of the COBAS® TaqMan® Negative Control, the HIV-1 Low Positive Control and the HIV-1 High Positive Control must be included in each test batch. The batch is valid if no flags appear for any of the controls [**HIV-1 H(+)C, HIV-1 L(+)C** and **CTM (–) C**].

There are no requirements regarding the position of the controls on the sample rack.

Check the batch printout for flags and comments to ensure that the batch is valid. Refer to the following manuals for detailed information on printing results and interpreting comments: (a) the AMPLILINK Software Version 3.1.x Series Application Manual for use with the COBAS® AmpliPrep Instrument, COBAS® TaqMan® Analyzer, and COBAS® TaqMan® 48 Analyzer and either (b) the COBAS® TaqMan® Analyzer (plus optional Docking Station) Instrument Manual for use with AMPLILINK Software, Version 3.1.x Series or (c) the COBAS® TaqMan® 48 Analyzer Instrument Manual for use with AMPLILINK Software, Version 3.1.x Series.

### Negative Control

The **CTM (–) C** must yield a "Target Not Detected" result. If the **CTM (–) C** is flagged as invalid, then the entire batch is invalid. Repeat the entire process (specimen and control preparation, amplification and detection). If **CTM (–) C** is consistently invalid in multiple batches, contact your local Roche office for technical assistance.

### Positive Controls

The assigned range for **HIV-1 L(+)C** and **HIV-1 H(+)C** is specific for each lot of reagents, and is provided on the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test reagent cassette barcodes.

The HIV-1 RNA cp/mL for **HIV-1 L(+)C** and **HIV-1 H(+)C** should fall within their assigned ranges. If one or both of the positive controls are flagged as invalid, then the entire batch is invalid. Repeat the entire process (specimen and control preparation, amplification and detection). If the HIV-1 RNA titer of one or both of the positive controls is consistently outside the assigned ranges in multiple batches, contact your local Roche office for technical assistance.

## PROCEDURAL PRECAUTIONS

1. As with any test procedure, good laboratory technique is essential to the proper performance of this assay.

## PROCEDURAL LIMITATIONS

1. This test has been validated for use with only human plasma collected in EDTA anticoagulant. Testing of other specimen types may result in inaccurate results.
2. The performance of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test has neither been evaluated with specimens containing HIV-1 groups O and N, nor with specimens containing HIV-2.
3. Reliable results are dependent on adequate specimen collection, transport, storage and processing procedures.
4. The presence of AmpErase enzyme in the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Master Mix reduces the risk of amplicon contamination. However, contamination from HIV-1 positive controls and clinical specimens can be avoided only by good laboratory practices and careful adherence to the procedures specified in this Package Insert.
5. Use of this product should be limited to personnel trained in the techniques of PCR.
6. This product can only be used with the COBAS® AmpliPrep Instrument and the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer.

## INTERFERING SUBSTANCES

Elevated levels of triglycerides, bilirubin, albumin, hemoglobin and human DNA in specimens as well as the presence of autoimmune diseases such as Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA) and Antinuclear Antibody (ANA) have been shown not to interfere with the quantitation of HIV-1 RNA or impact the specificity of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test.

The following drug compounds tested at the Peak Plasma Level ($C_{max}$) and at 3 times the $C_{max}$ have been shown not to interfere with the quantitation of HIV-1 RNA or impact the specificity of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test with the exception of Nevirapine, for which absence of interference has only been shown at the Peak Plasma Level ($C_{max}$):

| | |
|---|---|
| **Nucleotide DNA Polymerase Inhibitors**<br>Tenofovir<br>Adefovir dipivoxil | **Nucleoside Reverse Transcriptase and DNA Polymerase Inhibitors**<br>Lamivudine<br>Zidovudine<br>Stavudine<br>Abacavir<br>Didanosine |
| **HIV Protease Inhibitors**<br>Indinavir<br>Saquinavir<br>Ritonavir<br>Nelfinavir<br>Amprenavir<br>Lopinavir/Ritonavir | **Non-nucleoside HIV Reverse Transcriptase Inhibitors**<br>Nevirapine<br>Efavirenz<br><br>**HIV Fusion Inhibitor**<br>Enfuvirtide |
| **Immune Modulators**<br>Interferon alpha-2a<br>Interferon alpha-2b<br>Peginterferon alpha-2a<br>Peginterferon alpha-2a + Ribavirin<br>Interferon alpha-2b + Ribavirin | **Antidepressants**<br>Paroxetine HCl<br>Fluoxetine<br>Sertraline |
| **Nucleoside Inhibitors**<br>Ribavirin | **Compounds for the Treatment of Herpes Viruses**<br>Ganciclovir<br>Valganciclovir<br>Acyclovir |

## NON-CLINICAL PERFORMANCE EVALUATION

### A. Limit of Detection

The limit of detection of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test was determined by analysis of three independent dilution series of a HIV-1 Secondary Standard (prepared from the HIV-1B strain 8E5 LAV in HIV-1-negative human EDTA plasma). The concentration of the HIV-1 Secondary Standard is traceable to the WHO 1st International Standard for HIV-1 RNA for Nucleic Acid-Based Techniques (NAT) (NIBSC 97/656). A total of 108 replicates per concentration level were tested.

The concentration of HIV-1 RNA in EDTA plasma that can be detected with a positivity rate of greater than 95% as determined by probit analysis is 48 cp/mL. The study was performed for three lots of COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test reagents and the combined results are shown in Table 2.

***Table 2***
***Limit of Detection of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test determined with HIV-1B Secondary Standard in EDTA plasma***

| Level No. | HIV-1B Input Conc. (copies/mL) | Total Number of Replicates Tested | Number of Positives | Hit Rate |
|---|---|---|---|---|
| 1 | 100 | 108 | 108 | 100% |
| 2 | 75 | 108 | 108 | 100% |
| 3 | 50 | 108 | 107 | 99% |
| 4 | 40 | 108 | 99 | 92% |
| 5 | 30 | 108 | 98 | 91% |
| 6 | 20 | 108 | 81 | 75% |
| 7 | 10 | 108 | 55 | 51% |

### B. Precision

Precision and linearity of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test were determined by analysis of serial dilutions prepared from a highly concentrated cell culture stock of the HIV-1B in HIV-1-negative human EDTA plasma. The concentration assignment of the HIV-1B linearity panel was performed by a method that ensures traceability to the WHO 1st International Standard for HIV-1 RNA for Nucleic Acid-Based Techniques (NAT) (NIBSC 97/656).

Within-Run, Run-to-Run and Total Precision were evaluated in accordance with the methods defined in the CLSI Guideline EP5-A, "Evaluation of Precision Performance of Clinical Chemistry Devices".[41] A run, consisting of 5 dilution levels and 7 replicates at each level, was performed daily for 15 days. Each sample was carried through the entire COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test procedure, including specimen preparation, amplification and detection using different systems operated by multiple users. Therefore, the precision reported here represents all aspects of the test procedure. The study was performed for three lots of COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test reagents, and the results are shown in Table 3.

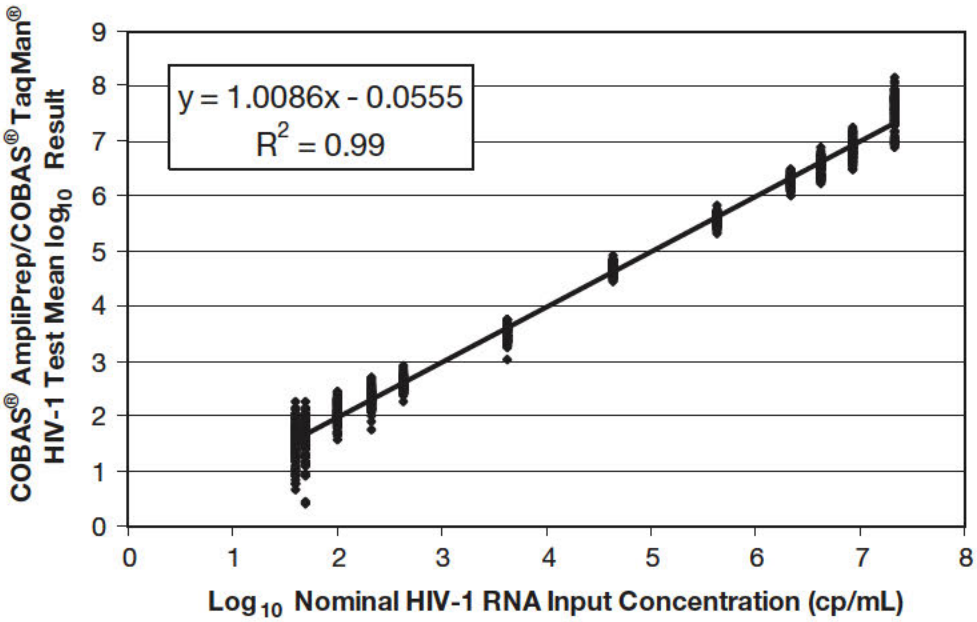
***Table 3***
***Total Precision for three lots of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test***

| Nominal HIV-1 Conc. Levels [cp/mL] | $Log_{10}$ of Nominal Conc. | Lot 1 | | Lot 2 | | Lot 3 | |
|---|---|---|---|---|---|---|---|
| | | Total CV [%] | Total precision as SD [$log_{10}$] | Total CV [%] | Total precision as SD [$log_{10}$] | Total CV [%] | Total precision as SD [$log_{10}$] |
| 1.0E+02 | 2.000 | 39.5 | 0.18 | 41.4 | 0.18 | 41.8 | 0.18 |
| 4.3E+03 | 3.634 | 24.5 | 0.11 | 29.3 | 0.13 | 23.1 | 0.10 |
| 4.3E+04 | 4.634 | 20.0 | 0.09 | 20.6 | 0.09 | 16.9 | 0.08 |
| 4.3E+05 | 5.634 | 22.4 | 0.10 | 20.0 | 0.09 | 22.8 | 0.10 |
| 2.2E+06 | 6.333 | 24.0 | 0.10 | 23.5 | 0.10 | 24.7 | 0.10 |

## C. Linear Range

As shown in Figure 7, the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test was found to give a linear response from 48 HIV-1 RNA cp/mL to 10,000,000 HIV-1 RNA cp/mL applying the accuracy acceptance criterion of ± 0.3 $\log_{10}$ from the nominal input concentration. The study was performed using one lot of COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test reagents, with 103 – 105 replicates per level.

***Figure 7***
***Linear range of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test***




## D. Inclusivity

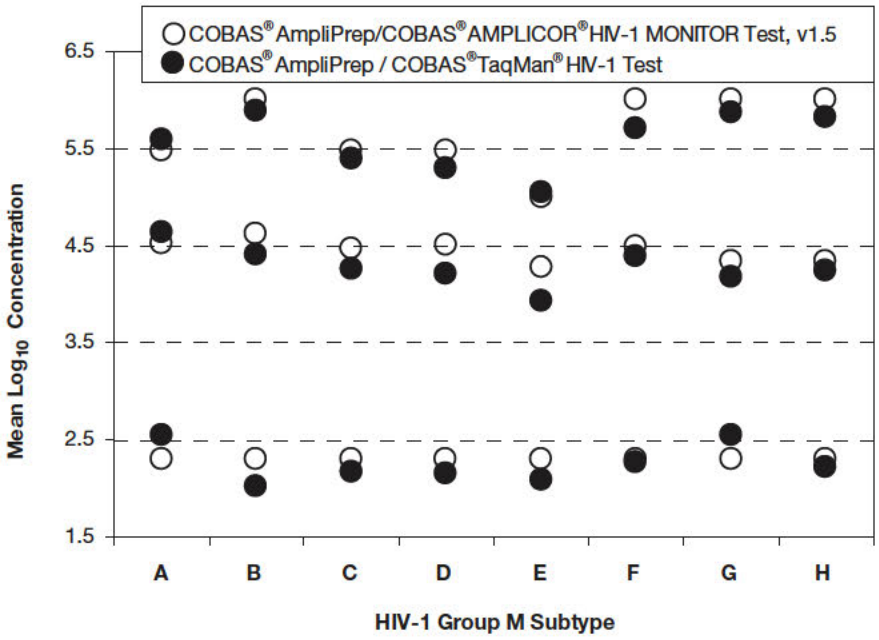
Eight subtype categories have been proposed for HIV-1 group M based on nucleotide divergence. These subtypes are designated with capital alphabetical letters from A through H[40].

Similar Subtype Quantitation

The performance of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test on HIV-1 subtypes was evaluated by analysis of cell culture stock material of representatives of each HIV-1 group M subtype A through H. The assignment of nominal concentrations of the cell culture stock solutions was performed by the COBAS® AmpliPrep/COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 assay method (Figure 8). Based on the determined stock concentrations, the HIV-1 target concentrations of 2.0E+02, 1.9 - 3.3E+04 and 1.0E+05 - 1.0E+06 cp/mL were prepared for each HIV-1 subtype by exact dilution of the cell culture stock solution in EDTA plasma. Afterwards the concentrations were determined by the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test in n=7 replicates per level of each subtype using one reagent lot. Results were compared to the input concentrations as determined by the reference method.

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test gave equivalent results for all tested representatives of the HIV-1 group M subtypes (see Figure 8). Mean observed $\log_{10}$ concentration results were within ± 0.3 $\log_{10}$ of the respective reference method input concentration.

***Figure 8***
***Performance of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test on HIV-1 group M Subtypes A through H compared against the reference method COBAS® AmpliPrep/COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5***




Subtype Limit of Detection

Parent solutions containing HIV-1 cell culture material representing HIV-1 subtypes A to H have been obtained as frozen stocks from German National Reference Centre for Retrovirology at University Erlangen-Nuremberg, Germany. The certified parent input concentration as determined by the VERSANT® HIV-1 RNA 3.0 Assay (bDNA) reference method was used to prepare the Subtype LOD-panels. Two independent dilution series of the different HIV-1 subtypes were evaluated on two days with one lot of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test in a total of 24 replicates per concentration level for each subtype representative. The results of the probit analyses at 95% hit rate demonstrate that the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test has a sensitivity of ≤ 50 cp/mL across all subtypes ranging from < 15 to 46 cp/mL of HIV-1 group M as shown in Table 4.

*Table 4*
*Inclusivity as Limit of Detection of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test as determined with cell-cultured viral stocks of the different HIV-1 subtypes diluted in EDTA plasma*

| Subtype | Isolate Designation | 95% hit rate conc. by probit model | 95% Confidence Interval [cp/mL] | | Lowest level with ≥ 95% hit rate |
|---|---|---|---|---|---|
| | | [cp/mL] | Lower | Upper | [cp/mL] |
| **A** | 92UG029 | 20 | | | **30** |
| **A** | 92UG037 | 52 | | | **50** |
| **A** | 4237A/98 | < 15 | | | **15** |
| **A combined** | 92UG029/92UG037/ 4237A/98 | **23** | **12** | **34** | **40** |
| **B** | HIV-1 Secondary Standard (8E5 LAV) | 38 | | | **40** |
| **B** | WHO 1st Int. Std. for HIV-1 RNA (97/656) | 46 | | | **50** |
| **B** | MVP-899-87 | 35 | | | **50** |
| **B combined** | HIV-1 Secondary Standard (8E5 LAV)/ WHO 1st Int. Std. for HIV-1 RNA (97/656)/ MVP-899-87 | **40** | **33** | **50** | **50** |
| **C** | 92BR025 | 16 | | | **30** |
| **C** | 98TZ017 | 50 | | | **50** |
| **C** | 3777A/97 | 16 | | | **15** |
| **C combined** | 92BR025/98TZ017/ 3777A/97 | **31** | **25** | **41** | **40** |
| **D** | 92UG021 | 35 | | | **40** |
| **D** | 92UG035 | < 15 | | | **15** |
| **D** | 92UG024 | 17 | | | **30** |
| **D combined** | 92UG021/92UG035/ 92UG024 | **26** | **22** | **34** | **40** |
| **E** | 92TH022 | < 15 | | | **15** |
| **E** | 92TH009 | 44 | | | **50** |
| **E** | 92TH001 | 34 | | | **50** |
| **E combined** | 92TH022/92TH009/ 92TH001 | **33** | **27** | **44** | **50** |
| **F** | 93BR020 | **46** | **34** | **82** | **40** |
| **G** | ARP173/RU570 | **< 15** | NA | NA | **15** |
| **H** | ARP175/HIV V1557 | **< 15** | NA | NA | **15** |

*NA - not applicable (no analysis possible since the statistical software does not allow calculating a confidence interval based on the observed hit rate profile)*

### E. Specificity

The clinical specificity of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test was determined by analysis of HIV-1-negative EDTA plasma specimens from blood donors. A total of 513 individual EDTA plasma specimens were tested. All specimens were negative for HIV-1 RNA. Based on these results, the clinical specificity of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is 100% with the confidence interval ranging from 99.3 to 100%.

## F. Analytical Specificity

The analytical specificity of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test was evaluated by adding cultured organisms (viruses, bacteria, yeast) or DNA (HTLV-2) at 5E+04 particles/mL input concentration into HIV-1-negative human EDTA plasma and into HIV-1-positive human EDTA plasma at 1E+04 cp/mL HIV-1 (see Table 5). The cultured organisms added to specimens have been shown not to interfere with the quantitation of HIV-1 RNA or impact the specificity of COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test.

***Table 5***
***Analytical Specificity Organisms***

| **Virus** | **Bacteria** |
|---|---|
| *Adenovirus type 2* | *Staphylococcus aureus* |
| *Cytomegalovirus* | *Propionibacterium acnes* |
| *Epstein-Barr Virus* | |
| *Human Herpes Virus type 6* | |
| *Herpes simplex virus type 1* | |
| *Herpes simplex virus type 2* | **Yeast** |
| *Human T-Cell Lymphotropic virus type 1* | *Candida albicans* |
| *Human T-Cell Lymphotropic virus type 2* | |
| *Influenza A* | |
| *Hepatitis A virus* | |
| *Hepatitis B virus* | |
| *Hepatitis C virus* | |

## G. Method Correlation

The performance of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test was compared to the COBAS® AmpliPrep/COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5, to the COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 and to the VERSANT HIV-1 RNA 3.0 Assay (bDNA) by analysis of n=71 undiluted clinically characterized plasma specimens from HIV-1 infected patients. Specimens were obtained from Teragenix (Fort Lauderdale, FL, USA). Correlation was determined using the specimens for which quantitative results were obtained with each method under comparison. Specimens with a concentration result above the measuring range of the COBAS® AmpliPrep/COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 using the ultrasensitive procedure (PHS) were prediluted to fall into the measuring range of the PHS. Bivariate linear regression analysis was performed on those specimens that yielded results within the linear range as shown in Figures 9, 10 and 11. The results obtained with the three methods under comparison showed high correlation.

***Figure 9***
***Correlation of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test and the COBAS® AmpliPrep/COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5***

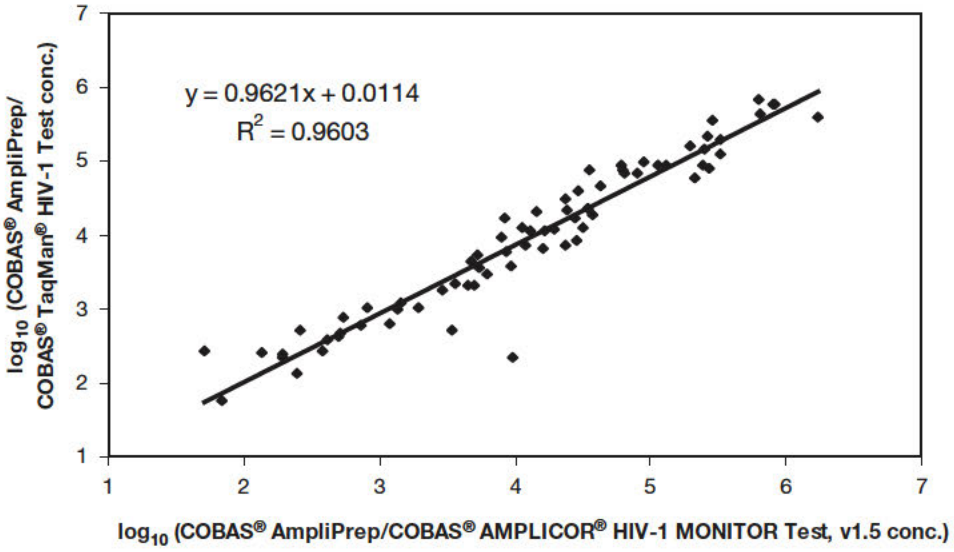


***Figure 10***
***Correlation of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test and the COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5***

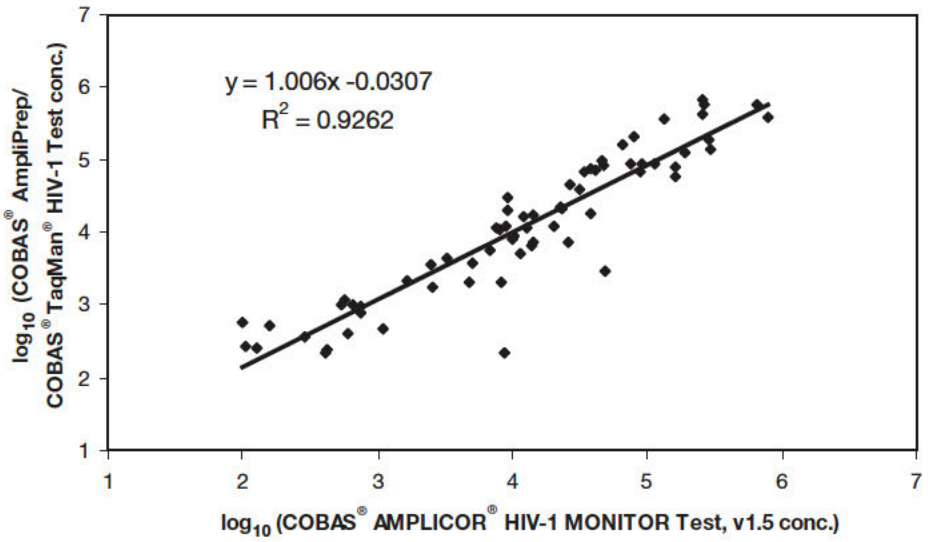
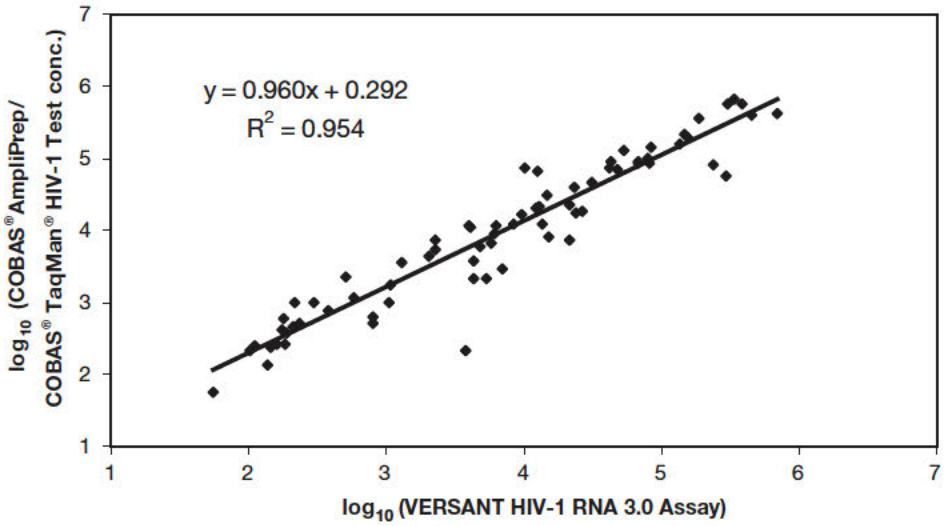



***Figure 11***
***Correlation of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test and the VERSANT HIV-1 RNA 3.0 Assay (bDNA)***



## CLINICAL PERFORMANCE EVALUATION

### A. Reproducibility

This study was conducted to evaluate the reproducibility of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test in EDTA plasma. Well characterized HIV-1 group M, subtype B virus stock cultures and EDTA plasma that was negative for HIV-1 RNA and HIV 1/2 antibodies were used to construct a 10-member panel. The study was designed to measure inter-run, intra-run, lot to lot, day to day, site to site, and operator to operator variability. Each panel was tested by multiple operators at each of three sites; one internal Roche Molecular Systems, Inc. (RMS) site, and two sites external to RMS. Each operator performed 5 days of testing on each of 3 lots of reagents with each panel. Each operator was to complete 1 run per day. Each run comprised a single panel with each panel member tested in duplicate.

Precision was evaluated using a random effects model with terms for lot, site/instrument, operator within site, between day/run and within-run components.

Table 6 shows the total precision variance and total precision standard deviation as determined by analysis of variance. Analysis of variance provides an estimate of the total precision of the test that properly weights the between-lot, between-site/instrument, between-operator, between-day/run (day-to-day) and within-run components. The total precision reported as lognormal variance was less than 35% for all panel members except the panel member with the lowest concentration (100 copies/mL). The within-run component contributed the most variability (56% to 100%), followed by lot-to-lot variability. The site/instrument, operator and day/run components contributed less to variability.

Table 7 summarizes the results for the HIV-1 Negative Panel Member. The negative panel member was used to estimate the analytical specificity of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test. One false positive result was observed out of 179 valid test results giving a specificity of 99% [95% Confidence Interval (CI) = (0.97, 1.00)].

***Table 6***
***Reproducibility Results Summary***

| HIV-1 RNA Concentration (log10 cp/mL) | | | Contribution to Total Variance (%) | | | | | Total Precision |
|---|---|---|---|---|---|---|---|---|
| Expected | Observed (Average) | N | Lot | Site / Instrument | Operator | Day / Run | Within Run | Standard Deviation (Lognormal %CV) |
| 2.000 | 2.020 | 147 | 0% | 0% | 0% | 0% | **100%** | 0.19 (46%) |
| 2.699 | 2.743 | 173 | 2% | 6% | 4% | 20% | **68%** | 0.14 (34%) |
| 3.000 | 2.995 | 178 | 0% | 8% | 0% | 8% | **84%** | 0.14 (32%) |
| 3.699 | 3.743 | 178 | 16% | 5% | 3% | 18% | **57%** | 0.14 (34%) |
| 4.301 | 4.410 | 179 | 14% | 6% | 2% | 5% | **74%** | 0.14 (32%) |
| 4.699 | 4.836 | 178 | 21% | 1% | 0% | 2% | **76%** | 0.12 (29%) |
| 5.398 | 5.501 | 178 | 32% | 0% | 0% | 6% | **62%** | 0.13 (31%) |
| 5.699 | 5.837 | 178 | 28% | 0% | 0% | 3% | **68%** | 0.14 (34%) |
| 6.699 | 6.871 | 108 | 32% | 4% | 0% | 9% | **56%** | 0.13 (30%) |

*Note: Within assay range results are from 4.80E+1 cp/mL to 1.00E+7 cp/mL inclusive.*

***Table 7***
***HIV-1 Negative Panel Member Summary***

| Total Valid Results | Target Not Detected | Target Detected | Analytical Specificity | Exact 95% CI |
|---|---|---|---|---|
| 179 | 178 | 1 | 0.99 | (0.97, 1.00) |

### Clinical Sensitivity and Specificity

#### Methodology

This study was designed to evaluate the clinical specificity and sensitivity of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test by testing fresh and frozen samples collected from normal healthy donors and patients with HIV-1. This study compared Test results obtained with the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test to those obtained with an FDA-approved test, ie, the COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5. Clinical specificity of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test was evaluated by testing 399 frozen samples and 120 fresh samples, in EDTA plasma, collected from normal healthy blood donors who were negative for HIV-1 antibodies. Frozen samples were randomly distributed across testing sites. Fresh samples were distributed to testing sites in a non-random manner. Clinical sensitivity of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test was evaluated testing 351 frozen samples with HIV-1 RNA concentrations ≥50 cp/mL and 122 fresh samples in EDTA plasma, collected from HIV-1-positive blood donors. Frozen samples were randomly distributed across testing sites, stratified by CD4 count category. Fresh samples were distributed to testing sites in a non-random manner. Frozen samples used in this study were previously tested with COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 and, therefore, were not retested in this study on that platform. Collection of frozen normal donor (HIV-1-negative) samples occurred at 4 sites: 1 each in Tennessee, Florida, Pennsylvania, and California. Collection of frozen HIV-1-positive samples occurred at 6 sites: 1 each in California, New Jersey, Maryland, and Missouri, and at 2 sites in Florida. Collection of fresh samples occurred at 5 sites, as follows: HIV-1-positive samples were collected at 4 sites: 2 in Florida, 2 in California; samples from normal healthy donors were collected at 1 site in Tennessee. Fresh samples collected for this study were tested prospectively on the COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 platform. Testing was performed at 3 sites, with 1 COBAS® AmpliPrep/COBAS® TaqMan® 48 Analyzer system per site, with at least 3 reagent lots.

#### Statistical Methods

Normal subjects were considered evaluable if they contributed both valid COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test and COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 results (where the COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 result was Target Not Detected). HIV-1 subjects were considered evaluable if they contributed both valid COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test and COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 results (where the COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 result was ≥50 cp/mL).

COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test clinical specificity was calculated as the percentage (95% exact confidence interval [CI]) of normal subjects with Target Not Detected COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 results, who had Target Not Detected COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test results. COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test clinical specificity was calculated overall and by sample type (fresh and frozen separately).

COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test clinical sensitivity was calculated as the percentage (95% exact CI) of HIV-1 subjects with COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 results ≥50 cp/mL, who had detectable HIV-1 viral load on the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test. COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test clinical sensitivity was calculated overall and by CD4 count category (<200, 200-500, >500 cells/µL) and sample type (fresh and frozen separately).

#### Results

Table 8 summarizes the number of fresh and frozen samples from evaluable normal and HIV-1 subjects in this study.

***Table 8***
***Number of Evaluable Normal and HIV-1 Subjects by Sample Type***

| Sample Type | Normal Subjects | HIV-1 Subjects | Total |
|---|---|---|---|
| Fresh | 120 (23.1%) | 122 (25.8%) | 242 |
| Frozen | 399 (76.9%) | 351 (74.2%) | 750 |
| Total | 519 | 473 | 992 |

Table 9 shows the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test clinical specificity results from the 519 evaluable normal subjects. The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test clinical specificity was 99.4% (516/519; 95% CI = 98.3% to 99.9%). The HIV-1-positive COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test results were all <40 cp/mL.

***Table 9***
***COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test Specificity - Evaluable Normal Subjects***

| COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 Result | COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test Result | | Total | COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test Clinical Specificity (95% Exact CI) |
|---|---|---|---|---|
| | HIV-1 Positive | HIV-1 Negative | | |
| HIV-1 Negative | 3 (0.6%) | 516 (99.4%) | 519 | 99.4% (98.3%, 99.9%) |

*Note: CI = Confidence Interval*

Table 10 shows the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test clinical sensitivity results from the 473 evaluable HIV-1 subjects. The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test clinical sensitivity was 98.3% (465/473; 95% CI = 96.7% to 99.3%).

***Table 10***
***COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test Sensitivity - Evaluable HIV-1 Subjects***

| COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 Result | COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test Result | | Total | COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test Clinical Sensitivity (95% Exact CI) |
|---|---|---|---|---|
| | HIV-1 Positive | HIV-1 Negative | | |
| HIV-1 Positive | 465 (98.3%) | 8 (1.7%) | 473 | 98.3% (96.7%, 99.3%) |

**All 8 samples that were determined to be positive for HIV-1 by COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 but not by COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test had a low copy number: 6 of the 8 had HIV-1 titers of < 100 cp/mL, 1 had a titer of 253 cp/mL, and the remaining sample had a titer of 479 cp/mL.*

*Note: CI = Confidence Interval*

Table 11 shows the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test clinical sensitivity by CD4 count category (<200, 200-500, >500 cells/µL).

***Table 11***
***COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test Sensitivity by CD4 Count Category - Evaluable HIV-1 Subjects***

| CD4 Count Category (cells/µL) | COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 Result | COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test Result | | Total | COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test Clinical Sensitivity (95% Exact CI) |
|---|---|---|---|---|---|
| | | HIV-1 Positive | HIV-1 Negative | | |
| <200 | HIV-1 Positive | 141 (99.3%) | 1 (0.7%) | 142 | 99.3% (96.1%, 100.0%) |
| 200 – 500 | HIV-1 Positive | 212 (98.6%) | 3 (1.4%) | 215 | 98.6% (96.0%, 99.7%) |
| >500 | HIV-1 Positive | 112 (96.6%) | 4 (3.4%) | 116 | 96.6% (91.4%, 99.1%) |

*Note: CI = Confidence Interval*

**Conclusion**

When compared with results from the COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5, clinical specificity of the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test was 99.4%, and clinical sensitivity was 98.3%, indicating similar performance of both tests. Furthermore, in both fresh and frozen samples, the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test showed similar specificity (>98%) and sensitivity (>98%), indicating comparable test performance for both sample types.

Selection of the HIV-1 samples included in this study was based on a single test result with the COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5. For low titer samples, the results from the COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 are subject to large variability (coefficient of variation = 50% at 100 cp/mL). All 8 samples that were determined to be positive for HIV-1 by the COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 but not by the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test had a low copy number: 6 of the 8 had HIV-1 titers of <100 cp/mL, 1 had a titer of 253 cp/mL, and the remaining sample had a titer of 479 cp/mL. The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test was able to detect 111 out of the 119 samples with titers <480 cp/mL. The observed results are consistent with what is expected with low-titer samples. Furthermore, the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test and the COBAS® AMPLICOR® HIV-1 MONITOR Test, v1.5 appeared to show comparable agreement across the linear range.

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test also showed similar clinical sensitivity among CD4 count categories (>96% overall), suggesting comparable performance in samples from HIV-1 patients with various CD4 counts.

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# ATTACHMENT 20

# AMPLICOR HIV-1 MONITOR™ TEST
**Standard/UltraSensitive**

AMPLICOR HIV-1 MONITOR Test US:83088 24 Tests

## INTENDED USE

The AMPLICOR HIV-1 MONITOR™ Test is an *in vitro* nucleic acid amplification test for the quantitation of Human Immunodeficiency Virus Type 1 (HIV-1) RNA in human plasma. The Test can be used with either the Standard or UltraSensitive Specimen Processing Procedure. When the Standard Specimen Processing Procedure is used, the Test can quantitate HIV-1 RNA over the range of 400 - 750,000 copies/mL. When the UltraSensitive Specimen Processing Procedure is used, the Test can quantitate HIV-1 RNA over the range of 50 - 75,000 copies/mL.

The Test is intended for use in conjunction with clinical presentation and other laboratory markers of disease progress for the clinical management of HIV-1 infected patients. The Test can be used to assess patient prognosis by measuring the baseline HIV-1 RNA level or to monitor the effects of antiretroviral therapy by measuring changes in plasma HIV-1 RNA levels during the course of antiretroviral treatment. Monitoring the effects of antiretroviral therapy by serial measurement of plasma HIV-1 RNA has been validated for patients with baseline viral loads ≥ 25,000 copies/mL.

The AMPLICOR HIV-1 MONITOR Test is not intended to be used as a screening test for HIV or as a diagnostic test to confirm the presence of HIV infection.

## SUMMARY AND EXPLANATION OF THE TEST

Human Immunodeficiency Virus (HIV) is the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS)[1-3]. HIV infection can be transmitted by sexual contact, exposure to infected blood or blood products, or by an infected mother to the fetus[4]. Within three to six weeks of exposure to HIV, infected individuals generally develop a brief, acute syndrome characterized by flu-like symptoms and associated with high levels of viremia in the peripheral blood[5-8]. In most infected individuals this is followed by an HIV-specific immune response and a decline of plasma viremia, usually within four to six weeks of the onset of symptoms[9,10]. After seroconversion, infected individuals typically enter a clinically stable, asymptomatic phase that can last for years[11-13]. The asymptomatic period is characterized by persistent, low level plasma viremia[14] and by a gradual depletion of CD4+ T lymphocytes, leading to severe immunodeficiency, multiple opportunistic infections, malignancies and death[15]. Although virus levels in the peripheral blood are relatively low during the asymptomatic phase of the infection, virus replication and clearance appear to be dynamic processes in which high rates of virus production and infection of CD4+ cells are balanced by equally high rates of virus clearance, death of infected cells and replenishment of CD4+ cells, resulting in relatively stable levels of both plasma viremia and CD4+ cells[16-18].

Quantitative measurements of HIV viremia in the peripheral blood have shown that higher virus levels may be correlated with increased risk of clinical progression of HIV disease, and that reductions in plasma virus levels may be associated with decreased risk of clinical progression[19-21]. Virus levels in the peripheral blood can be quantitated by measurement of the HIV p24 antigen in serum, by quantitative culture of HIV from plasma, or by direct measurement of viral RNA in plasma using nucleic acid amplification or signal amplification technologies [22-26].

p24 antigen is the principle core protein of HIV and is found in serum either free or bound by anti-p24 antibody. Free p24 antigen can be measured with commercially available enzyme immunoassays (EIA), although the usefulness of p24 antigen as a marker of viral load is limited since the antigen is detectable in only 20% of asymptomatic patients and 40-50% of symptomatic patients. Procedures to dissociate antigen-antibody complexes improve the sensitivity of the p24 antigen tests, but the viral protein remains undetectable in most asymptomatic patients[22].

Infectious HIV in plasma can be cultured by inoculation into activated peripheral blood mononuclear cells (PBMC) from normal donors. Quantitation is achieved by inoculating PBMC with serial dilutions of the plasma specimen. Quantitative culture has limited utility for monitoring virus levels in infected individuals since only a small fraction of virus particles is infectious *in vitro*. Infectious virus is often undetectable in asymptomatic individuals[22].

HIV RNA in plasma can be quantitated by nucleic acid amplification technologies, such as the Polymerase Chain Reaction (PCR)[27-29]. The AMPLICOR HIV-1 MONITOR Test uses PCR technology to achieve maximum sensitivity and dynamic range for the quantitative detection of HIV-1 RNA in ACD or EDTA anti-coagulated plasma[24].

## PRINCIPLES OF THE PROCEDURE

The AMPLICOR HIV-1 MONITOR Test is based on five major processes: specimen preparation: reverse transcription (RT) of target RNA to generate complementary DNA (cDNA); PCR amplification of target cDNA using HIV-1 specific complimentary primers; hybridization of the amplified products to oligonucleotide probes specific to the target(s); and detection of the probe-bound amplified products by colorimetric determination.

The AMPLICOR HIV-1 MONITOR Test permits the reverse transcription and amplification of HIV-1 and Quantitation Standard (QS) RNA to occur simultaneously. The Master Mix reagent contains a biotinylated primer pair specific for HIV-1 and QS target nucleic acid.

The quantitation of HIV-1 viral RNA is performed using a Quantitation Standard (QS). The QS is a non-infectious RNA transcript that contains the identical primer binding sites as the HIV-1 target and a unique probe binding region that allows QS amplicon to be distinguished from HIV-1 amplicon. The QS is incorporated into each individual specimen at known copy number and is carried through the specimen preparation, reverse transcription, PCR amplification, hybridization and detection steps along with the HIV-1 target and is amplified along with the HIV-1 target. HIV-1 RNA levels in the test specimens are determined by comparing the absorbance of the specimen to the absorbance obtained for the Quantitation Standard. Therefore, the QS compensates for any effects of inhibition and controls for the amplification process to allow the accurate quantitation of each specimen.

### Specimen Preparation

The AMPLICOR HIV-1 MONITOR Test can be used with either of two specimen processing procedures, the Standard procedure and the UltraSensitive procedure. With the Standard specimen processing procedure, HIV-1 RNA can be quantitated over the range of 400 - 750,000 copies/mL. With the UltraSensitive procedure, HIV-1 RNA can be quantitated over the range of 50 - 75,000 copies/mL.

In the Standard specimen processing procedure, HIV-1 RNA is isolated directly from plasma by lysis of virus particles with a chaotropic agent followed by precipitation of the RNA with alcohol. With the UltraSensitive specimen processing procedure, HIV-1 viral particles are first pelleted from

the plasma specimen by high speed ultracentrifugation followed by lysis of the pelleted virus particles with a chaotropic agent and precipitation of the HIV-1 RNA with alcohol. A known number of QS RNA molecules is introduced into each specimen with the lysis reagent. The QS is carried through the specimen preparation, amplification and detection steps and is used for the quantitation of HIV-1 RNA in the test specimen. The QS compensates for any effects of inhibition to permit the accurate quantitation of HIV-1 RNA in each specimen.

**Reverse Transcription and PCR Amplification**

The AMPLICOR HIV-1 MONITOR Test amplifies and detects a 142 base target sequence located in a highly conserved region of the HIV-1 *gag* gene[30], defined by the primers SK431 and SK462. The *gag* region encodes the group-specific antigens or core structural proteins of the virion. The HIV-1 *gag* genes are generally about 1500 nucleotides in length and are located at the approximate positions 789-2290 in the HIV genome.

The reverse transcription and amplification reactions are performed with the thermostable recombinant enzyme *Thermus thermophilus* DNA Polymerase (r*Tth* pol). In the presence of manganese and under the appropriate buffer conditions, r*Tth* pol has both reverse transcriptase (RT) and DNA polymerase activity[31]. This allows both reverse transcription and PCR amplification to occur in the same reaction mixture.

Reverse Transcription

The processed specimens are added to the amplification mixture in reaction tubes in which both reverse transcription and PCR amplification occur. The downstream or antisense primer (SK431) and the upstream or sense primer (SK462) are biotinylated at the 5' ends. The reaction mixture is heated to allow the downstream primer to anneal specifically to the HIV-1 and QS target RNA. In the presence of excess deoxynucleoside triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine and thymidine triphosphates, r*Tth* pol extends the annealed primer forming a complementary (cDNA) strand.

PCR Amplification

Following reverse transcription of the HIV-1 and QS target RNA, the reaction mixture is heated to denature the RNA:cDNA hybrid and expose the HIV-1 and QS target sequences. As the mixture cools, the upstream primer anneals to the cDNA strand, the r*Tth* pol catalyzes the extension reaction, and a second DNA strand is synthesized. This completes the first cycle of PCR yielding a double stranded DNA copy (or amplicon) of each HIV-1 or QS RNA The reaction mixture is heated again to separate the resulting double-stranded DNA and expose the primer target sequences. As the mixture cools, the primers anneal to the target DNA. r*Tth* pol, in the presence of excess dNTPs, extends the annealed primers along the target templates to produce a 142 base pair sequence termed an **amplicon**. This process is repeated for a number of cycles, each cycle effectively doubling the amount of amplicon. Amplification occurs only in the region of the HIV-1 genome between the primers. The entire HIV-1 genome is not amplified.

Selective Amplification

Selective amplification of target nucleic acid from the clinical specimen in the AMPLICOR HIV-1 MONITOR Test is achieved by the use of AmpErase® and deoxyuridine triphosphate (dUTP). AmpErase (uracil-N-glycosylase, UNG) recognizes and catalyzes the destruction of DNA strands containing deoxyuridine[32], but not DNA containing thymidine. Deoxyuridine is not present in naturally occurring DNA, but is always present in amplicon due to the use of deoxyuridine

triphosphate as one of the dNTPs in the Master Mix reagent; therefore, only amplicon contain deoxyuridine. Deoxyuridine renders contaminating amplicon susceptible to destruction by AmpErase prior to amplification of the target DNA. AmpErase, which is included in the Master Mix Reagent, catalyzes the cleavage of deoxyuridine containing DNA at deoxyuridine residues by opening the deoxyribose chain at the C1-position. When heated in the first thermal cycling step at the alkaline pH of Master Mix, the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. AmpErase is inactive at temperatures above 55°C, i.e., throughout the thermal cycling steps, and therefore does not destroy target amplicon. Following amplification, any residual enzyme is denatured by the addition of the denaturation solution, thereby preventing the degradation of target amplicon. AmpErase in the AMPLICOR HIV-1 MONITOR Test has been demonstrated to inactivate at least $10^3$ copies of deoxyuridine-containing HIV-1 amplicon per PCR.

**Hybridization Reaction**

Following PCR amplification, the HIV-1 and QS amplicons are chemically denatured to form single stranded DNA by the addition of denaturation solution, and aliquots are added to separate wells of a microwell plate (MWP) coated with HIV-1-specific (SK102) and QS-specific (CP35) oligonucleotide probes. HIV-1 and QS amplicons are bound to HIV-1 and QS wells, respectively, by hybridization to the MWP-bound probes. To achieve quantitative results over a large dynamic range, serial dilutions of the denatured amplicon are analyzed in the MWP.

**Detection Reaction**

Following the hybridization reaction, the MWP is washed to remove any unbound material, and an avidin-horseradish peroxidase conjugate (Av-HRP) is added to each well of the MWP. The Av-HRP binds to the biotin-labeled amplicon captured by the plate-bound oligonucleotide probes. The MWP is washed again to remove unbound Av-HRP and a substrate solution containing hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB) is added to the wells. In the presence of hydrogen peroxide, the bound horseradish peroxidase catalyzes the oxidation of TMB to form a color complex. The reaction is stopped by addition of a weak acid, and the optical density at 450 nm is measured using an automated microwell plate reader.

**HIV-1 RNA QUANTITATION**

The AMPLICOR HIV-1 MONITOR Test quantitates viral load by utilizing a second target sequence (QS) that is added to the amplification mixture at a known concentration. The QS is a non-infectious 219 base *in vitro* transcribed RNA molecule with primer binding regions identical to those of the HIV-1 target sequence. The QS, therefore, contains SK431 and SK462 primer binding sites and generates an amplification product of the same length (142 bp) and base composition as the HIV-1 target. The probe binding region of the QS has been modified to differentiate QS-specific amplicon from HIV-1 target amplicon.

Within the linear range of the assay, the optical density (OD) in each well of the MWP is proportional to the amount of HIV-1 or QS amplicon in the well, and the total optical density is proportional to the amount of HIV-1 or QS RNA, respectively, input into each reverse transcription/ PCR amplification reaction. The amount of HIV-1 RNA in each specimen is calculated from the ratio of the total optical density for the HIV-1-specific well to the total optical density for the QS-specific well and the input number of QS RNA molecules using the following equation:

$$\frac{\text{Total HIV-1 OD}}{\text{Total QS OD}} * \text{Input HIV-1 QS copies / PCR reaction} * \text{Dilution Factor} = \text{HIV-1 RNA copies/mL}$$

Where:

| | |
|---|---|
| Total HIV-1 OD = | calculated total OD for HIV-1 amplicon |
| Total QS OD = | calculated total OD for QS amplicon |
| Input HIV-1 QS copies/PCR = | the number of copies of QS in each reaction; this information is lot specific and is entered by user |
| Dilution Factor = | factor to convert copies/PCR to copies/mL<br>• Dilution Factor = 40 for the Standard Procedure<br>• Dilution Factor = 4 for the UltraSensitive Procedure |

## REAGENTS

**AMPLICOR HIV-1 MONITOR Test** **24 Tests**
(US:83088)

**HIV-1 MONITOR Specimen Preparation Reagents**

**HIV-1 LYS** 2 x 9.0 mL
(HIV-1 MONITOR Lysis Reagent)
Tris HCl buffer
68% Guanidine thiocyanate
3% Dithiothreitol
< 1% Glycogen

Xn ✖ 68% (w/w) guanidine thiocyanate
Harmful

**HIV-1 QS** 2 x 0.1 mL
(HIV-1 MONITOR Quantitation Standard)
Tris-HCl buffer
< 0.001% Non-infectious *in vitro* transcribed RNA (microbial) containing HIV-1 primer binding sequences and a unique probe binding region
< 0.005 % Poly rA RNA (synthetic)
EDTA
Amaranth dye
0.05% Sodium azide

**HIV-1 DIL** 2 x 4.8 mL
(HIV-1 MONITOR Specimen Diluent)
Tris-HCl buffer
< 0.005 % Poly rA RNA (synthetic)
EDTA
0.05% Sodium azide

## AMPLICOR HIV-1 MONITOR Control Reagents

**NHP** — 2 x 1.6 mL
[Negative Plasma (Human)]
Human plasma, non-reactive by FDA licensed tests for antibody to HCV, antibody to HIV-1/2, and HBsAg
0.1% ProClin® 300

**HIV-1 (–) C** — 2 x 0.05 mL
(HIV-1 MONITOR (–) Control)
Tris-HCl buffer
< 0.005% Poly rA RNA (synthetic)
EDTA
0.05% Sodium azide

**HIV-1 L(+)C** — 2 x 0.05 mL
(HIV-1 MONITOR Low (+) Control)
Tris-HCl buffer
<0.001% Non-infectious *in vitro* transcribed RNA (microbial) containing HIV-1 sequences
<0.005% Poly rA RNA (synthetic)
EDTA
0.05% Sodium azide

**HIV-1 H(+)C** — 2 x 0.05 mL
(HIV-1 MONITOR High (+) Control)
Tris-HCl buffer
<0.001% Non-infectious *in vitro* transcribed RNA (microbial) containing HIV-1 sequences
<0.005% Poly rA RNA (synthetic)
EDTA
0.05% Sodium azide

## AMPLICOR HIV-1 MONITOR Amplification Reagents

**HIV-1 MMX** — 2 x 0.5 mL
(HIV-1 MONITOR Master Mix)
Bicine buffer
Glycerol
<0.01% r*Tth* DNA Polymerase (r*Tth* pol, microbial)
Potassium acetate
<0.07% dATP, dCTP, dGTP, TTP, dUTP
<0.001% SK431 and SK462 primers, biotinylated
<0.01% AmpErase® (microbial)
0.05% Sodium azide

**HIV-1 $Mn^{2+}$** 2 x 0.1 mL
(HIV-1 MONITOR Manganese Solution)
2% Manganese acetate
Acetic acid
Amaranth dye
0.05% Sodium azide

**AMPLICOR HIV-1 MONITOR Detection Reagents**

**HIV-1 MONITOR MWP** 2 x 12 Tests
(HIV-1 MONITOR Microwell Plate)
MWP coated with HIV-1 specific DNA probe (Rows A to F) and
QS-specific probe (Rows G to H)
Twelve, 8-well strips in one resealable pouch with desiccant

**[1] MONITOR DN** 1 x 12 mL
(MONITOR Denaturation Solution)
1.6% sodium hydroxide
EDTA
Amaranth dye

Xi 1.6% (w/w) sodium hydroxide
Irritant

**[2] MONITOR HYB** 2 x 12 mL
(MONITOR Hybridization Buffer)
Sodium phosphate solution
< 25% Sodium thiocyanate
< 0.2% solubilizer

**[3] AV-HRP** 2 x 12 mL
(Avidin-Horseradish Peroxidase Conjugate)
Tris HCl buffer
<0.001% Avidin-horseradish peroxidase conjugate
Bovine gamma globulin (mammalian)
Emulsit 25 (Dai-ichi Kogyo Seiyaku Co., Ltd)
0.1% Phenol
1% ProClin 150

**[4A] SUB A** 2 x 12 mL
(Substrate A)
Citrate solution
0.01% Hydrogen peroxide
0.1% ProClin 150

**[4B] SUB B** 2 x 3 mL
(Substrate B)
0.1% 3,3',5,5'-Tetramethylbenzidine (TMB)
40% Dimethylformamide (DMF)

T 40% (w/w) Dimethylformamide (DMF)
Toxic

R: 61-20/21-36 May cause harm to the unborn child. Harmful by inhalation and in contact with the skin. Irritating to eyes
S: 53-46 Avoid exposure - obtain special instructions before use. In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).

**[5] STOP** 2 x 12 mL
(Stop Reagent)
4.9% sulfuric acid

**10X WB** 3 x 90 mL
(10X-Wash Concentrate)
< 2% Phosphate buffer
< 9% Sodium chloride
EDTA
< 2% Detergent
0.5% ProClin

## WARNINGS AND PRECAUTIONS

A. **FOR *IN VITRO* DIAGNOSTIC USE.**

B. This test is for use with human plasma collected in EDTA or ACD anticoagulants only. ***Heparin has been shown to inhibit PCR and must not be used with this procedure.***

C. Do not pipette by mouth.

D. Do not eat, drink or smoke in laboratory work areas. Wear disposable gloves, laboratory coats and eye protection when handling specimens and kit reagents. Wash hands thoroughly after handling specimens and kit reagents.

E. Avoid microbial and ribonuclease contamination of reagents when removing aliquots from reagent bottles. The use of sterile disposable pipettes and pipette tips is recommended.

F. Do not pool reagents from different lots or from different bottles of the same lot.

G. Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.

H. Do not use a kit after its expiration date.

I. Material Safety Data Sheets (MSDS) are available on request from the Roche Response Center® or your local Roche office. See telephone numbers listed on the last page of this insert.

J. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Pre-Amplification Area and moving to the Post-Amplification (Amplification/Detection) Area. Pre-amplification activities must begin with reagent preparation and proceed to specimen preparation. Supplies and equipment must be dedicated to each activity and not used for other activities or moved between areas. Gloves must be worn in each area and must be changed before leaving that area. Equipment and supplies used for reagent preparation must not be used for specimen preparation activities or for pipetting or processing amplified DNA or other sources of target DNA. Post-Amplification supplies and equipment must be confined to the Post-Amplification Area at all times.

K. Specimens should be handled as if infectious using safe laboratory procedures such as those outlined in *Biosafety in Microbiological and Biomedical Laboratories*[33] and in the NCCLS Document M29-T[34]. Thoroughly clean and disinfect all work surfaces with 10% bleach.

L. **CAUTION:** This kit contains a component (**NHP**) derived from human blood. The source material has been assayed by US FDA approved tests and found non-reactive for the presence of Hepatitis B Surface Antigen (HBsAg) and antibodies to HIV-1-1/2 and HCV. No known test methods can offer complete assurance that product derived from human blood will not transmit infectious agents. Therefore all human sourced material should be considered potentially infectious. **NHP** should be handled as if infectious using safe laboratory procedures such as those outlined in *Biosafety in Microbiological and Biomedical Laboratories*[33] and in the NCCLS Document M29-T[34]. Thoroughly clean and disinfect all work surfaces with 10% bleach.

***Note:*** ***Commercial liquid household bleach typically contains sodium hypochlorite at a concentration of 5.25%. A 1:10 dilution of household bleach will produce a 0.5% sodium hypochlorite solution.***

M. **HIV-1 QS, HIV-1 DIL, HIV-1 MMX, HIV-1 $Mn^{2+}$, HIV-1 (–) C, HIV-1 L(+)C,** and **HIV-1 H(+)C** contain sodium azide. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. After disposing of sodium azide containing solutions down laboratory sinks, flush the drains with a large volume of water to prevent azide buildup.

N. Wear eye protection, laboratory coats and disposable gloves when handling **HIV-1 LYS, HIV-1 MMX, [1] MONITOR DN, [2] MONITOR HYB, [3] AV-HRP, [4A] SUB A, [4B] SUB B,** Working Substrate (mixed **[4A] SUB A and [4B] SUB B**) and **[5] STOP**. Avoid contact of these materials with the skin, eyes or mucous membranes. If contact does occur, immediately wash with large amounts of water. Burns can occur if left untreated. If spills of these reagents occur, dilute with water before wiping dry.

O. Avoid contact between the skin or mucous membranes and **[4B] SUB B** or Working Substrate. If skin contact occurs wash immediately with large amounts of water.

P. **[4B] SUB B** and Working Substrate contain dimethylformamide which has been reported to be toxic in high oral doses and may be harmful to the unborn child. Skin contact, inhalation of fumes and ingestion should be avoided. If skin contact occurs, wash thoroughly with soap and water and seek medical advice immediately.

Q. Do not allow **HIV-1 LYS**, which contains guanidine thiocyanate, to contact sodium hypochlorite (bleach) solution. This mixture can produce a highly toxic gas.

R. Screw-cap tubes must be used for specimen and control preparation to prevent splashing and potential cross-contamination of specimens. ***Do not use snap cap tubes.***

## STORAGE AND HANDLING REQUIREMENTS

A. ***Do not freeze reagents.***

B. Store **HIV-1 LYS, HIV-1 QS,** and **HIV-1 DIL** at 2-8°C. Unopened, these reagents are stable until the expiration date indicated. Once opened, any unused portion should be discarded.

C. A precipitate forms in **HIV-1 LYS** during storage at 2-8°C . Prior to use, warm to 25-37°C and mix thoroughly to dissolve the precipitated material. Once opened, any unused portion should be discarded. Working Lysis Reagent (prepared by addition of **HIV-1 QS** to **HIV-1 LYS**) should be stored at room temperature and used within 4 hours of preparation.

D. Store **HIV-1 MMX** and **HIV-1 $Mn^{2+}$** at 2-8°C. These reagents are stable until the expiration date indicated. Working Master Mix (prepared by the addition of **HIV-1 $Mn^{2+}$** to **HIV-1 MMX**) must be stored at 2-8°C and is stable for 4 hours at 2-8°C.

E. Store **NHP, HIV-1 (–) C, HIV-1 L(+)C** and **HIV-1 H(+)**C at 2-8°C. These reagents are stable until the expiration date indicated.

F. Store **[1] MONITOR DN, [2] MONITOR HYB** and **[5] STOP** at 2-25°C. These reagents are stable until the expiration date indicated.

G. Store **HIV-1 MONITOR MWP** at 2-8°C in the foil pouch provided. **HIV-1 MONITOR MWP** is stable in the unopened pouch until the expiration date indicated. Once opened, **HIV-1 MONITOR MWP** is stable for 3 months (or until the expiration date, whichever comes first) in the resealed pouch containing desiccant.

H. Store **[3] AV-HRP, [4A] SUB A** and **[4B] SUB B** at 2-8°C. Unopened, these reagents are stable until the expiration date indicated. Once opened, these reagents are stable for 3 months (or until the expiration date, whichever comes first).

I. Working Substrate must be freshly prepared by mixing **[4A] SUB A** with **[4B] SUB B** and is stable at ambient temperature for 3 hours when protected from light. Do not expose **[4A] SUB A, [4B] SUB B** or Working Substrate (mixed **[4A] SUB A** and **[4B] SUB B**) to metals, oxidizing agents or direct light.

J. Store **10X WB** at 2-25°C. The solution is stable until the expiration date indicated. Working Wash Solution (1X) should be stored at 2-25°C in a clean, closed plastic container and is stable for 2 weeks.

## MATERIALS PROVIDED

**AMPLICOR HIV-1 MONITOR Test**
(US:83088)

**A. AMPLICOR HIV-1 MONITOR Specimen Preparation Reagents**

**HIV-1 LYS**
(HIV-1 MONITOR Lysis Reagent)
**HIV-1 QS**

(HIV-1 MONITOR Quantitation Standard)

**HIV-1 DIL**
(HIV-1 MONITOR Specimen Diluent)

**B. AMPLICOR HIV-1 MONITOR Control Reagents**

**NHP**
[Negative Plasma (Human)]

**HIV-1 (–) C**
(HIV-1 MONITOR (–) Control)

**HIV-1 L(+)C**
(HIV-1 MONITOR Low (+) Control)

**HIV-1 H(+)C**
(HIV-1 MONITOR High (+) Control)

**C. AMPLICOR HIV-1 MONITOR Amplification Reagents**

**HIV-1 MMX**
(HIV-1 MONITOR Master Mix)

**HIV-1 $Mn^{2+}$**
(HIV-1 MONITOR Manganese Solution)

**D. AMPLICOR HIV-1 MONITOR Detection Reagents**

**HIV-1 MONITOR MWP**
(HIV-1 MONITOR Microwell Plate)

**[1] MONITOR DN**
(MONITOR Denaturation Solution)

**[2] MONITOR HYB**
(MONITOR Hybridization Buffer)

**[3] AV-HRP**
(Avidin-Horseradish Peroxidase Conjugate)

**[4A] SUB A**
(Substrate A)

**[4B] SUB B**
(Substrate B)

**[5] STOP**
(Stop Reagent)

**10X WB**
(10X-Wash Concentrate)

**MATERIALS REQUIRED BUT NOT PROVIDED:**

**Pre-Amplification Area - Reagent Preparation**

- For PE Applied Biosystems GeneAmp® PCR System 9600 thermal cycler, use MicroAmp® Reaction Tubes (PE #N801-0533), Caps (PE #N801-0535), Tray/Retainers (PE #N801-0530) and Base (PE #N801-0531)
- For PE Applied Biosystems GeneAmp PCR System 2400 thermal cycler, use MicroAmp Reaction Tubes (PE #N801-0533), Caps (PE #N801-0535), Tray/Retainers (PE #N801-5530) and Base (PE #N801-5531)
- Plastic resealable bag
- Eppendorf® Repeater® pipet with 1.25 mL Combitip® Reservoir (sterile, individually wrapped)
- Pipettors (capacity 50 µL and 200 µL)* with aerosol barrier or positive displacement RNase-free tips
- Vortex mixer
- Latex gloves, powderless

**Pre-Amplification Area - Specimen and Control Preparation**

- 2.0 mL screw-cap tubes, sterile (Sarstedt 72.693.005, 72.694.005, 72.694.006, or equivalent) **
- 1.5 mL screw-cap tubes, sterile (Sarstedt 72.692.105, or equivalent) **
- Tube racks (Sarstedt 93.1428)
- Ethyl alcohol, absolute or 95% (freshly diluted to 70% using deionized water)
- Isopropyl alcohol, reagent grade (Fisher A416-500)
- Fine tip, sterile transfer pipets, RNase-free
- Sterile disposable, polystyrene pipets (5 mL, 10 mL and 25 mL)
- Micropipettes (capacity 12.5 µL, 25 µL, 50 µL, 100 µL, 200 µL, 400 µL, 500 µL, 600 µL, 800 µL and 1000 µL)* with aerosol barrier or positive displacement tips
- Microcentrifuge (max. RCF 16,000 x g, min. RCF 12,500 x g); Eppendorf 5415C, HERMLE Z230M, or equivalent
- Refrigerated centrifuge and fixed angle rotor (45 degrees, capacity for at least 24 1.5 mL tubes) with a RCF of 23,600 x g: Heraeus 17RS with HFA 22.1 rotor, or equivalent..
- Vortex mixer
- Latex gloves, powderless

**Post Amplification Area - Amplification/Detection**

- Multichannel pipettor (capacity 25 µL and 100 µL) or electronic pipettor (Impact® or AMPLICOR®)
- Aerosol barrier or positive displacement pipettor tips (25 µL and 100 µL) and barrier-free tips (100 µL)*
- PE Applied Biosystems GeneAmp PCR System 9600 or GeneAmp PCR System 2400 thermal cycler
- MicroAmp base and cap installing tool for PE Applied Biosystems GeneAmp PCR System 9600 or GeneAmp PCR System 2400
- Microwell Plate Washer***
- Microwell Plate Reader****
- Disposable Reagent Reservoirs
- Microwell plate lid (Fisher catalog no. 07-200-376)
- Incubator 37°C ± 2°C
- Distilled or deionized water
- Graduated vessels
- Latex gloves, powderless

* Pipettors should be accurate within 3% of stated volume. Aerosol barrier or positive displacement RNase-free tips must be used where specified to prevent specimen and amplicon cross-contamination.

** Screw-cap tubes must be used for specimen and control preparation to prevent splashing and potential cross-contamination of specimens. ***Do not use snap cap tubes.***

*** Capable of washing 12 x 8 microwell format with 250-300 µL of Wash Solution per well at 30 second time intervals.

**** Microwell Reader Specifications: Bandwidth = 10 ± 3 nm; Absorbance Range = 0 to ≥ 3.00 $A_{450}$; Repeatability ≤1%; Accuracy ≤ 3% from 0 to 2.00 $A_{450}$; Drift ≤ 0.01 $A_{450}$ per hour.

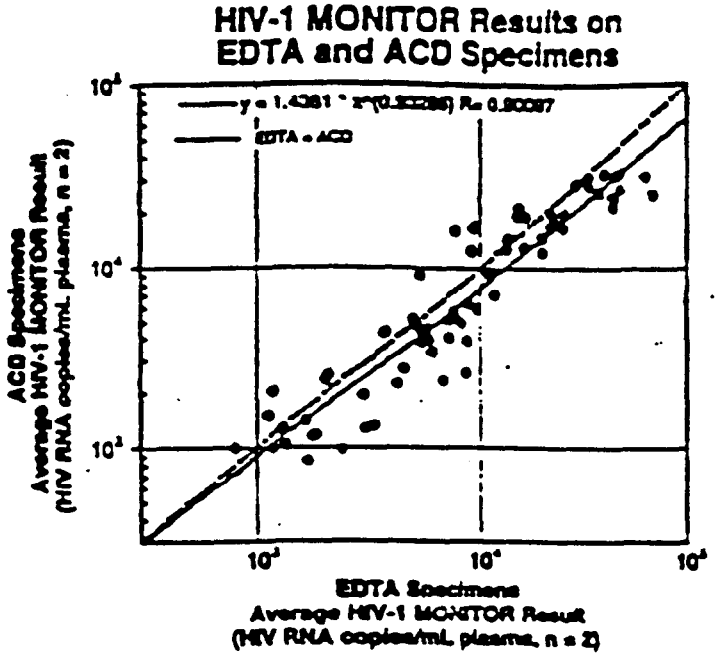
**SPECIMEN COLLECTION, STORAGE and TRANSPORT**

***NOTE: Handle all specimens as if they are capable of transmitting infectious agents.***

**A. Specimen Collection**

The AMPLICOR HIV-1 MONITOR Test is for use with plasma specimens only. Blood should be collected in sterile tubes using EDTA (lavender top, Becton-Dickinson #6454 or equivalent) or ACD (yellow top, Becton-Dickinson #4606 or equivalent) as the anticoagulant. ***Specimens anticoagulated with heparin are unsuitable for this test.*** The use of ACD anticoagulated specimens will yield test results that are approximately 15% lower than test results obtained from EDTA anticoagulated specimens due to the dilution effect of the 1.5 mL of ACD anticoagulant present in the blood collection tube. Figure I shows the effect of ACD and EDTA anticoagulants on HIV RNA results from plasma specimens.

*Figure 1*
*Comparison of ACD and EDTA Anticoagulants*


HIV-1 MONITOR Results on
EDTA and ACD Specimens
EDTA = ACD
ACD Specimens
Average HIV-1 MONITOR Result
(HIV RNA copies/mL plasma, n = 2)
EDTA Specimens
Average HIV-1 MONITOR Result
(HIV RNA copies/mL plasma, n = 2)

Store whole blood at 2-25°C for no longer than 6 hours. Do not refrigerate. Separate plasma from whole blood within 6 hours of collection by centrifugation at 800-1600 x g for 20 minutes at room temperature. Transfer plasma to a sterile polypropylene tube.

## B. Specimen Storage

Plasma specimens may be stored at room temperature for up to 1 day, at 2-8°C for up to 5 days, or frozen at -20°C to -80°C.

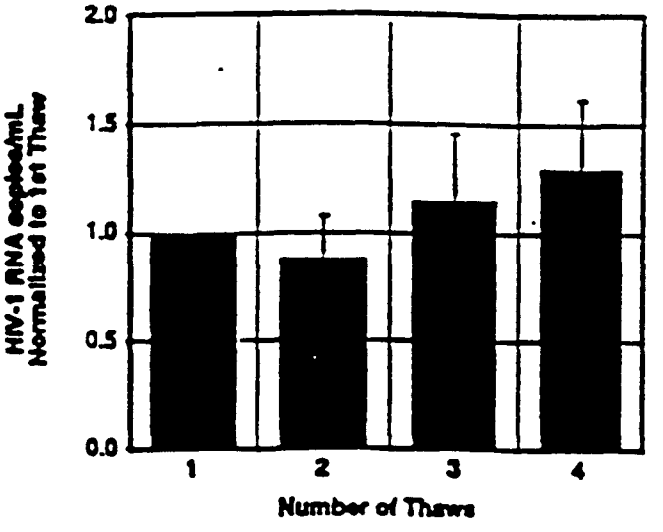
Virus pellets obtained from step 9 of the UltraSensitive Specimen Preparation Procedure are stable for up to 6 hours at room temperature and at least 14 days at -20°C or colder.

It is recommended that specimens be stored in 600-700 µL aliquots in sterile, 2 mL polypropylene screw cap tubes (such as Sarstedt catalog no. 782.694.006). In-house studies have shown that plasma specimens may be frozen and thawed up to 3 times. Figure 2 shows the data from these freeze-thaw studies.

## C. Specimen Transport

Transportation of whole blood or plasma must comply with country, federal, state and local regulations for the transport of etiologic agents[35]. Whole blood must be transported at 2-25°C and processed within 6 hours of collection. Plasma may be transported at 2-8°C or frozen.

*Figure 2*
*Average HIV-1 Results from Three Pools of HIV-1 Positive Plasma*
*After 2, 3 and 4 Freeze-Thaw Cycles*




**INSTRUCTIONS FOR USE**

***Note:*** ***Visually examine reagents for sufficient reagent volume before beginning the test procedure.***

***Note:*** ***Serum and plasma specimens must be at ambient temperature before use. Use pipettors with aerosol barrier or positive displacement tips where specified. Use extreme care to ensure selective amplification.***

***Note:*** ***Screw-cap tubes must be used for specimen and control preparation to prevent splashing and potential cross-contamination of specimens. Do not use snap cap tubes.***

**Run Size:**

Each kit contains reagents sufficient for two 12-test batches, which may be performed separately or simultaneously. It is recommended that one replicate of the HIV-1 MONITOR (-) Control, the HIV-1 MONITOR Low (+) Control and the HIV-1 MONITOR High (+) Control be included in each test run (see *"Quality Control"* section).

The Specimen Preparation and Amplification Reagents are packaged in 12-test, single-use bottles. For the most efficient use of reagents, specimens and controls should be processed in batches of 12 or 24.

**Workflow:**

The AMPLICOR HIV-1 MONITOR Test can be completed in one day or over two days. If the testing is to be completed in a single work day, follow the instructions in Parts A through D in order. If the testing is to be completed over 2 days, the procedure may be stopped after Specimen Preparation (Part B) or after Amplification (Part C).

- To perform specimen processing on Day 1 and amplification/detection on Day 2, perform "Standard Specimen and Control Preparation", Steps B1.1 through B1.17, or "UltraSensitive

Specimen and Control Preparation". Steps B2.1 through B2.22, on Day 1 and store the processed specimens as indicated in Step B1.17 or Step B2.22, respectively. On Day 2 begin with Part A, thaw the processed specimens at room temperature and then continue with either Step B1.18 (for Standard Specimen Processing) or Step B2.23 (for UltraSensitive Specimen Processing).

- To complete specimen processing and amplification on Day 1 and detection on Day 2, perform Parts A, B and C on Day 1 and store the denatured amplicon as indicated in Step C.5. Continue with Part D on Day 2.

**A. Reagent Preparation.**
**Performed in: Pre-Amplification - Reagent Preparation Area**

***Note:*** ***Store HIV-1 MMX and HIV-1 $Mn^{2+}$ at 2 - 8°C until use.***

1. Place each batch of up to 12 MicroAmp reaction tubes in a single row of a MicroAmp tray. Lock tubes in place with a tube retainer.
2. Prepare Working Master Mix by adding 100 µL **HIV-1 $Mn^{2+}$** to one tube of **HIV-1 MMX** (the mixture is sufficient for 12 reactions). *It is not necessary to measure the volume of Master Mix. Add 100 µL **HIV-1 $Mn^{2+}$** to the entire tube of **HIV-1 MMX**.* **Recap the HIV-1 MMX tube and mix well by inverting the tube 10-15 times** The pink dye in **HIV-1 $Mn^{2+}$** is used for visual confirmation that **HIV-1 $Mn^{2+}$** has been added to **HIV-1 MMX**. ***Discard remaining HIV-1 $Mn^{2+}$.***
3. Pipette 50 µL of Working Master Mix into each reaction tube using a repeat pipettor or a pipettor with an aerosol barrier or positive displacement tip. Confirm that **HIV-1 $Mn^{2+}$** was added to **HIV-1 MMX** by checking for the pink color. ***Discard unused Working Master Mix.***
4. Place the MicroAmp tray in a plastic, resealable bag, and move to the Pre-Amplification - Specimen Preparation Area. ***Store the MicroAmp Tray at 2-8°C until specimen preparation is completed. Amplification must begin within 4 hours of preparation of the Working Master Mix.***
5. Remove gloves and dispose of them properly. Remove labcoat and/or any other protective garments and leave them in the Reagent Preparation Area.

**B. Specimen and Control Preparation**
**Performed in Pre-Amplification-Specimen and Control Preparation Area**

***Note:*** ***All reagents must be at ambient temperature before use.***

***Note:*** ***To amplify previously prepared specimens, perform the steps in Part A, thaw prepared specimens at room temperature and continue with Part B1, "Standard Specimen and Control Preparation", Step B1.18, or with Part B2, "UltraSensitive Specimen and Control Preparation", Step B2.23.***

***Note:*** ***A precipitate forms in HIV-1 LYS upon storage at 2-8°C. Prior to use, warm at 25-37°C and mix to dissolve the precipitated material.***

B1. Standard Specimen and Control Preparation

1. Prepare 70% ethanol. For 12 tests, mix 11.0 mL 95% ethanol and 4.0 mL of deionized or distilled water or mix 14.0 mL absolute ethanol with 6.0 mL of deionized or distilled water.
2. Label a 2.0 mL screw cap microcentrifuge tube for each specimen and control. ***DO NOT USE SNAP CAP TUBES***. See "*Warnings and Precautions*" section.
3. Prepare Working Lysis Reagent. Vortex **HIV-1 QS** for at least 10 seconds prior to use to ensure that it is thoroughly mixed. For each batch of up to 12 specimens and controls, add 100 µL **HIV-1 QS** to one bottle of **HIV-1 LYS** and mix well. *It is not necessary to measure the volume of **HIV-1 LYS**. Add 100 µL **HIV-1 QS** to the entire bottle of **HIV-1 LYS***. The pink dye in **HIV-1 QS** is used for visual confirmation that **HIV-1 QS** has been added to **HIV-1 LYS**. ***Discard remaining HIV-1 QS.***
4. Thaw plasma specimens at room temperature and vortex for 3 - 5 seconds.
5. Spin tube briefly to collect specimen in base of tube. Take care to avoid contaminating gloves when manipulating specimens.
6. Dispense 600 µL Working Lysis Reagent into each tube. Check that Working Lysis Reagent is pink to confirm that **HIV-1 QS** was added to **HIV-1 LYS**.
7. Add 200 µL of each patient specimen to appropriately labeled tubes. Cap the tubes and vortex for 3 - 5 seconds.
8. For each negative, low and high positive control, add 200 µL **NHP** to the appropriate tubes. Cap the tubes and vortex for 3 - 5 seconds, then add 50 µL of **HIV-1 (–)C, HIV-1 L(+)C** and **HIV-1 H(+)C** to the appropriately labeled tubes. Cap the tubes and mix.
9. Incubate the tubes for 10 minutes at room temperature.
10. Add 800 µL room temperature 100% isopropanol to each tube. Re-cap the tube and vortex for 3 - 5 seconds.
11. Put an orientation mark on each tube and place the tubes into the microcentrifuge with the orientation mark facing outward, so that the pellet will align with the orientation mark. Centrifuge specimens at maximum speed (at least 12,500 x g) for 15 minutes at room temperature.
12. Using a new, fine tip disposable transfer pipette for each tube, carefully remove and discard the supernatant from each tube, being careful not to disrupt the pellet (which may not be visible). Remove as much liquid as possible without disturbing the pellet. Withdraw the supernatant slowly, allowing the liquid to drain completely off the sides of the tube. ***Do not use vacuum aspiration***.
13. Add 1.0 mL room temperature 70% ethanol to each tube, re-cap the tube, and vortex for 3 - 5 seconds.
14. Place the tubes into a microcentrifuge with the orientation marks facing outward and centrifuge the tubes for 5 minutes at maximum speed (at least 12,500 x g) at room temperature.
15. Carefully remove the supernatant using a new, fine tip disposable transfer pipette for each tube as before without disturbing the pellet. The pellet should be clearly visible at this step. ***Remove as much of the supernatant as possible! Residual ethanol can inhibit the amplification***.
16. Add 400 µL **HIV-1 DIL** to each tube. Re-cap the tube and vortex vigorously for 10 seconds to resuspend the extracted RNA. Note that insoluble material often remains.

17. Amplify the processed specimens (Step B.18) within 2 hours of preparation or store frozen at -20°C or colder for up to one week.

18. Pipette 50 µL of each prepared control and patient specimen to the appropriate MicroAmp reaction tubes using a micropipettor with plugged tips. Use a new tip for each specimen and control. Cap the tubes. Seal the caps using the MicroAmp cap installing tool. Record the positions of the controls and specimens.

19. ***Transfer the MicroAmp Tray with sealed tubes containing the processed specimens and controls in Working Master Mix to the Amplification/Detection Area.***

## B2. Specimen and Control Preparation - UltraSensitive Procedure

1. Pre-cool the ultracentrifuge and rotor to 2 - 8°C. See the operating instructions for the ultracentrifuge for details.

2. Prepare 70% ethanol. For 12 tests, mix 11.0 mL of 95% ethanol and 4.0 mL of deionized or distilled water or mix 14.0 mL absolute ethanol and 6.0 mL of deionized or distilled water.

3. Prepare Working Lysis Reagent. Vortex **HIV-1 QS** for at least 10 seconds prior to use to ensure that it is thoroughly mixed. For each batch of up to 12 specimens and controls, add 25 µL **HIV-1 QS** to one bottle of **HIV-1 LYS** and mix well. *It is not necessary to measure the volume of **HIV-1 LYS***. Add 25 µL **HIV-1 QS** to the entire bottle of **HIV-1 LYS.** The pink dye in **HIV-1 QS** is used for visual confirmation that **HIV-1 QS** has been added to **HIV-1 LYS**. ***Discard the remaining HIV-1 QS.***

4. Label a 1.5 mL screw cap microcentrifuge tube for each specimen and control. ***DO NOT USE SNAP CAP TUBES.*** See *"Warnings and Precautions"* section.

5. Thaw plasma specimens at room temperature and vortex for 3 - 5 seconds.

6. Spin tubes briefly to collect specimens in base of the tubes. Take care to avoid contaminating gloves when manipulating specimens.

7. Add 500 µL of each patient specimen to the appropriately labeled tube.

8. For each negative and positive control, add 500 µL NHP to each appropriately labeled tube.

9. Put an orientation mark on each tube and place the tubes into the ultracentrifuge with the orientation mark facing outward, so that the pellets will align with the orientation marks. Centrifuge specimens and control tubes containing NHP at 23,600 x g at 2 - 8° C for 60 minutes.

10. Using a new, fine tip disposable transfer pipette for each tube, carefully remove and discard the supernatant from each tube, being careful not to disrupt the pellet. ***The pellet may not be visible at this step. Remove as much liquid as possible without disturbing the pellet. Withdraw the supernatant slowly, allowing the liquid to drain completely off the sides of the tube. Do not use vacuum aspiration.***

11. Dispense 600 µL Working Lysis Reagent into each tube. Check that Working Lysis Reagent is pink to confirm that **HIV-1 QS** was added to **HIV-1 LYS**.

12. For each negative and positive control, add 12.5 µL of **HIV-1 (-)C, HIV-1 L(+)C**, and **HIV-1 H(+)C** to the appropriately labeled tubes. Cap the tubes and mix by vortexing for 3 - 5 seconds.

13. Incubate the tubes for 10 minutes at room temperature.

14. Add 600 µL room temperature 100% isopropanol to each tube. Re-cap the tube and vortex for 3 - 5 seconds

15. Place the tubes into the microcentrifuge with the orientation marks facing outward, so that the pellets will align with the orientation marks. Centrifuge specimens at maximum speed (at least 12,500 x g) for 15 minutes at room temperature.

16. Using a new, fine tip disposable transfer pipette for each tube, carefully remove and discard the supernatant from each tube, being careful not to disrupt the pellet. ***The pellet may not be visible at this step. Remove as much liquid as possible without disturbing the pellet. Withdraw the supernatant slowly, allowing the liquid to drain completely off the sides of the tube. Do not use vacuum aspiration.***

17. Add 1.0 mL room temperature 70% ethanol to each tube, re-cap the tube, and vortex for 3 - 5 seconds.

18. Place the tubes into the microcentrifuge with the orientation marks facing outward and centrifuge the tubes for 5 minutes at maximum speed (at least 12,500 x g) at room temperature.

19. Using a new, fine tip disposable transfer pipette for each tube, carefully remove and discard the supernatant from each tube, being careful not to disrupt the pellet. ***The pellet should be clearly visible at this step. Remove as much liquid as possible without disturbing the pellet. Withdraw the supernatant slowly, allowing the liquid to drain completely off the sides of the tube. Do not use vacuum aspiration.***

20. Repeat Step B2.19 to remove as much of the remaining supernatant as possible. Residual ethanol can inhibit the amplification.

21. Add 100 µL **HIV-1 DIL** to each tube. Re-cap the tube and vortex vigorously for 10 seconds to resuspend the extracted RNA. Note that insoluble material often remains.

22. Amplify the processed specimens (Step B.42) within 2 hours of preparation or store frozen at - 20°C or colder for up to one week.

23. Pipette 50 µL of each processed control and patient specimen to appropriate MicroAmp reaction tubes using a micropipettor with plugged tips. Use a new tip for each specimen and control. Cap the tubes. Seal the caps using the MicroAmp cap installing tool. Record the positions of the controls and specimens.

24. ***Transfer the MicroAmp tray with sealed tubes containing the processed specimens and controls in Working Master Mix to the Amplicfication/Detection Area.***

## C. Reverse Transcription and Amplification
**Performed in Post-Amplification - Amplification/Detection Area**

***Note: Warm the MONITOR DN to ambient temperature before use.***

***Note: Turn on the GeneAmp PCR System 9600 or GeneAmp PCR System 2400 thermal cycler at least 30 minutes prior to beginning the amplification.***

1. Place the Tray/Retainer assembly into the thermal cycler block.

2. Program the Perkin-Elmer GeneAmp PCR System 9600 or GeneAmp PCR System 2400 thermal cycler as follows.

| | |
|---|---|
| HOLD Program | 2 minutes at 50°C |
| HOLD Program | 30 minutes at 60°C |
| CYCLE Program (4 Cycles) | 10 sec at 95°C, 10 sec at 55°C, 10 sec at 72°C |
| CYCLE Program (26 Cycles) | 10 sec at 90°C, 10 sec at 60°C, 10sec at 72°C |
| HOLD Program | 15 minutes at 72°C |

In the CYCLE programs, the ramp time and allowed setpoint error should be left at the default settings of 0:00 (which is the maximum rate) and 2°C, respectively. Link the 5 programs together into a METHOD program.

Consult the PE Applied Biosystems GeneAmp PCR System 9600 or GeneAmp PCR System 2400 User's Manual for additional information on programming and operation of the thermal cycler.

3. Start the METHOD program. The program runs approximately one hour and 30 minutes.

4. Remove the tray from the thermal cycler at any time during the final HOLD program, place in the MicroAmp Base and continue immediately with Step C.5. Do not allow the reaction tubes to remain in the thermal cycler beyond the end of the final HOLD program and do not extend the final HOLD program beyond 15 minutes. ***DO NOT BRING AMPLIFIED SAMPLES INTO THE PRE-AMPLIFICATION AREA. AMPLIFIED CONTROLS AND SPECIMENS SHOULD BE CONSIDERED A MAJOR SOURCE OF CONTAMINATION.***

5. Remove the caps from the reaction tubes carefully to avoid creating aerosols of the amplification products. Immediately pipette 100 µL **[1] MONITOR DN** into each reaction tube using a multichannel pipettor and mix by pipetting up and down 5 times. (Program 1, AMPLICOR Electronic Impact Pipettor; see instructions for the AMPLICOR Electronic Impact Pipettor).

   Denatured amplicon can be held at room temperature no more than 2 hours before proceeding to the detection reaction (Part D). If the detection reaction can not be performed within 2 hours, re-cap the tubes and store the denatured amplicon at 2-8°C for up to one week.

**D. Detection**

Performed in: Amplification/Detection Area

***Note: Warm all reagents to room temperature prior to use.***

1. Prepare Working Wash Solution as follows. Examine **10X WB**, and, if necessary, warm at 30 - 37°C to redissolve any precipitate. Add 1 volume **10X WB** to 9 volumes of distilled or deionized water. Mix well. The volume of Working Wash Solution required depends on model of MWP washer being used. Store Working Wash Solution in a clean, closed plastic container at 2 - 25°C for up to 2 weeks.

2. Allow **HIV-1 MONITOR MWP** to warm to room temperature before removing from the foil pouch. Add 100 µL **[2] MONITOR HYB** to each well (Program 2, AMPLICOR Electronic Impact Pipettor; see instructions for the AMPLICOR Electronic Impact Pipettor). Rows A through F of the **HIV-1 MONITOR MWP** are coated with the HIV-specific oligonucleotide probe; rows G and H are coated with the QS-specific oligonucleotide probe.

3. Add 25 µL of the denatured amplicon to the HIV wells in row A of the MWP and mix up and down 10 times with a 12-channel pipettor with plugged tips. Make serial 5-fold dilutions in the HIV wells in rows B through F as follows. Transfer 25 µL from row A to row B and mix as before. Continue through row F. Mix row F as before, then remove and discard 25 µL. Discard pipet tips. (Addition of the denatured amplicon to the MWP and the serial dilutions may be done with Program 3 of the AMPLICOR Electronic Impact Pipettor. This program transfers 25 µL, mixes by pipetting 60 µL up and down 5 times, and aspirates 25 µL; see instructions for the AMPLICOR Electronic Impact Pipettor.)

4. Add 25 µL of the denatured amplicon to the QS wells in row G of the MWP and mix up and down 10 times with a 12-channel pipettor with plugged tips. Using a 12-channel pipettor with plugged tips, pipette 25 µL of denatured amplicon into row G of the MWP and mix by pipetting up and down 10 times (or use Program 3, AMPLICOR Electronic Impact Pipettor). Transfer 25 µL from row G to row H. Mix as before, then remove 25 µL from row H and discard.
5. Cover the MWP with MWP lid and incubate for 1 hour at 37°C ± 2°C.
6. Wash the MWP 5 times with the Working Wash Solution using an automated MWP washer. Program the MWP washer as follows:
   (a) Fill each well to top (250-300 µL). Let soak for 30 seconds. Aspirate.
   (b) Repeat Step (a) four additional times.
   (c) Tap the plate dry.
7. Add 100 µL **[3] AV-HRP** conjugate to each well (Program 2, AMPLICOR Electronic Impact Pipettor). Cover the MWP and incubate for 15 minutes at 37°C ± 2°C.
8. Wash the MWP as described in Step D.7.
9. Prepare the Working Substrate Solution. For each MWP, mix 12 mL **[4A] SUB A** with 3 mL **[4B] SUB B**. Protect Working Substrate from direct light. Working Substrate must be at room temperature and used within 3 hours of preparation.
10. Pipette 100 µL Working Substrate Solution into each well (Program 2, AMPLICOR Electronic Impact Pipettor).
11. Allow color to develop for 10 minutes at room temperature in the dark.
12. Add 100 µL **[5] STOP** to each well (Program 2, AMPLICOR Electronic Impact Pipettor).
13. Measure the optical density at 450 nm (single wavelength) within 10 minutes of **[5] STOP** addition.

**RESULTS**

For each specimen and control, calculate the HIV-1 RNA level as follows:

1. Choose the appropriate HIV well as follows:
   a. The HIV wells in rows A through F represent neat and 1:5, 1:25, 1:125, 1:625 and 1:3125 serial dilutions, respectively, of the HIV-1 amplicon. The absorbance values should decrease with the serial dilutions, with the highest $OD_{450}$ for each specimen or control in row A and the lowest $OD_{450}$ in row F.
   b. Choose the well with the lowest $OD_{450}$ that is ≥ 0.20 and ≤ 2.00 OD units.
   c. If any of the following conditions exist, see ***Unexpected Results***:
      - all HIV OD values < 0.20
      - all HIV OD values > 2.0
      - HIV OD values not in sequence (OD values do not decrease from well A to well F)
2. Subtract background from the selected HIV OD value (background = 0.07 OD units).
3. Calculate the **Total HIV OD** by multiplying the **background-corrected OD** value of the selected HIV well by the dilution factor associated with that well.

| Row | Dilution Factor |
|---|---|
| A | 1 |
| B | 5 |
| C | 25 |
| D | 125 |
| E | 625 |
| F | 3125 |

4. Choose the appropriate QS well as follows:

   a. The QS wells in rows G and H represent neat and 1:5 dilutions, respectively, of the QS amplicon. The absorbance value in row G should be greater than the value in row H.

   b. Choose the well with the lowest $OD_{450}$ that is ≥ 0.30 and ≤ 2.0 OD units.

   c. If the following conditions exist, see ***Unexpected Results:***

   - both QS OD values < 0.30
   - both QS OD values > 2.0
   - QS OD values not in sequence (well H has a higher OD than well G)

***NOTE:*** *All patient specimens and controls should yield QS OD values that meet the criteria described in Step 4, demonstrating that the specimen processing, reverse transcription, amplification and detection steps were performed correctly. If any control has a QS OD value that does not meet the criteria described in Step 4,* ***the entire run is invalid.*** *If any specimen has a QS OD value that does not meet the criteria described in Step 4,* ***the result for that specimen is invalid, but the run is still acceptable.***

5. Subtract background from the selected QS OD values (background = 0.07 OD units).

6. Calculate the ***Total QS OD*** by multiplying the ***background-corrected OD*** value of the selected QS well by the dilution factor associated with that well.

| Row | Dilution Factor |
|---|---|
| G | 1 |
| H | 5 |

7A. Determination of Standard Test Results

Calculate HIV-1 RNA copies/mL plasma as follows:

$$\frac{\text{Total HIV-1 OD.}}{\text{Total QS OD}} * \text{Input HIV-1 QS copies / PCR reaction} * 40 = \text{HIV-1 RNA copies/mL}$$

Where:

| | | |
|---|---|---|
| Total HIV-1 OD | = | calculated Total HIV-1 OD (from Step 3) |
| Total QS OD | = | calculated Total QS OD (from Step 6) |
| Input HIV-1 QS copies/PCR | = | the number of copies of QS in each reaction; this information is lot specific. See AMPLICOR HIV-1 MONITOR Test Data Card. |
| 40 | = | factor to convert copies per PCR to copies per mL of plasma |

*NOTE: Test results greater than 750,000 should be reported as "Greater than 750,000 copies/mL". If quantitative results are desired for such specimens, the original plasma specimen should be diluted with HIV negative human plasma and retested. Test results less than 400 should be reported as "HIV-1 RNA detected; Less than 400 copies/mL".*

7B. Determination of UlraSensitive Test Results

Calculate HIV-1 RNA copies/mL plasma as follows:

$$\frac{\text{Total HIV-1 OD}}{\text{Total QS OD}} * \text{Input HIV-1 QS copies / PCR reaction} * 4 = \text{HIV-1 RNA copies/mL}$$

Where:

| | | |
|---|---|---|
| Total HIV-1 OD | = | calculated Total HIV-1 OD (from Step 3) |
| Total QS OD | = | calculated Total QS OD (from Step 6) |
| Input HIV-1 QS copies/PCR | = | the number of copies of QS in each reaction; this information is lot specific. See AMPLICOR HIV-1 MONITOR Test Data Card. |
| 4 | = | factor to convert copies per PCR to copies per mL of plasma |

*NOTE: Test results greater than 75,000 should be reported as "Greater than 75,000 copies/mL". If quantitative results are desired for such specimens, the original plasma specimen should be retested using the Standard Specimen Processing Procedure. Test results less than 50 should be reported as "HIV-1 RNA detected; Less than 50 copies/mL".*

8. Unexpected Results

a. ***All HIV OD values < 0.20.*** Do not calculate these results. Report these results as: "HIV-1 RNA not detected (Less than 400 copies/mL)" for the Standard procedure and "HIV-1 RNA not detected (Less than 50 copies/mL)" for the UltraSensitive procedure.

b. ***All HIV OD values > 2.0.*** If all HIV-1 wells have OD values greater than 2.0, the HIV-1 copy number is above the linear range of the assay. Do not calculate these results. Report the result as "Not determined". If quantitative results are desired, retest the specimen as follows:

- If the test result was generated using the Standard specimen processing procedure, prepare a 1:50 dilution of the original specimen with HIV negative human plasma and repeat the test using the Standard procedure. Calculate the HIV-1 result as above, then multiply the final result by 50.
- If the test result was generated using the UltraSensitive specimen processing procedure, retest the original specimen undiluted using the Standard specimen processing procedure.

c. ***HIV OD values out of sequence.*** If HIV wells do not follow the general pattern of decreasing OD values from well A to well F, an error in dilution may have occurred. Examine the data using the criteria shown in (g) below to determine if an error occurred. If an error occurred, the result for that specimen is invalid and the entire test procedure for that specimen (including specimen preparation) should be repeated.

d. ***Both QS OD values < 0.30.*** If both QS wells have OD values less than 0.30 then either the processed specimen was inhibitory to the amplification or the RNA was not recovered during specimen processing. The result for that specimen is invalid. Repeat the entire test procedure (including specimen processing) for that specimen.

e. ***Both QS OD values > 2.0.*** If both QS wells have OD values greater than 2.0, an error occurred. The result for that specimen is invalid. Repeat the entire test procedure (including specimen processing) for that specimen.

f. ***QS OD values out of sequence.*** QS OD values out of sequence. If the absorbance of well H is greater than the absorbance in well G, then an error occurred. The result for that specimen is invalid. Repeat the entire test procedure (including specimen processing) for that specimen.

g. ***Examples of unexpected results.***

- The OD values for HIV wells should follow a pattern of decreasing OD values with increasing Dilution Factor (i.e., from well A to well F), except for wells that are saturated and wells with background OD values.
- In reactions containing high HIV-1 RNA copies per mL, wells A, B and C can become saturated, turning a greenish-brown color prior to the addition of Stop Solution and a brown color after the addition of Stop Solution, resulting in lower $OD_{450}$ (see examples 1 and 2 in Table 1). These results are valid even though the HIV wells do not have decreasing OD values from well A through well F.
- In reactions containing low HIV-1 RNA copies/mL, wells B through F may contain background OD values (see example 3 in Table 1). Such tests are valid even though the HIV wells do not have decreasing OD values from well A through well F. Wells with OD values > 2.3 may be saturated and wells with very low OD values (OD < 0.1) are close to background. These wells may not follow a pattern of decreasing OD values from well A to well F (examples 1, 2 and 3).
- All wells with OD values ≤ 2.3 and ≥ 0.1 should follow a pattern of decreasing OD values from well A to well F. If OD values ≤ 2.3 and ≥ 0.1 do not follow a pattern of decreasing OD values from well A to well F, then an error occurred (example 4). The results for this specimen are invalid. Repeat the entire test procedure (including specimen processing) for that specimen.

***Table 1***
***Examples of Unexpected Results***

| Specimen Results ($OD_{450}$) | | | | | |
|---|---|---|---|---|---|
| Row | Dilution Factor | Example 1 | Example 2 | Example 3 | Example 4 |
| A | 1 | 2.610 | 2.564 | 0.812 | 3.126 |
| B | 5 | 2.461 | 2.684 | 0.161 | 0.857 |
| C | 25 | 3.112 | 2.432 | 0.055 | 1.432 |
| D | 125 | 2.668 | 1.032 | 0.064 | 0.292 |
| E | 625 | 2.984 | 0.287 | 0.079 | 0.074 |
| F | 3125 | 1.568 | 0.074 | 0.052 | 0.066 |
| Interpretation: | | Very high titer specimen. Not an error | High titer specimen. Not an error | Low titer specimen. Not an error | Error |

## QUALITY CONTROL

It is recommended that one replicate of the AMPLICOR HIV-1 (–) Control, the AMPLICOR HIV-1 MONITOR Low (+) Control, and the AMPLICOR HIV-1 MONITOR High (+) Control be included in each test run. As with any new laboratory procedure, new operators should consider the use of additional controls each time the test is performed until such time as a high degree of confidence is reached in their ability to perform the test correctly. There are no requirements regarding the position of the controls in the MicroAmp tray.

All controls should yield QS OD values that meet the criteria described in Step 4 of the ***RESULTS*** section, demonstrating that the specimen processing, reverse transcription, amplification and detection steps were performed correctly. ***If any control has a QS OD value that does not meet the criteria described in RESULTS, Step 4, the entire run is invalid.*** Repeat the entire process (specimen and control preparation, reverse transcription, amplification and detection).

### Negative Control

The AMPLICOR HIV-1 (–) Control should yield a "Not Detected" result, i.e., all HIV-1 OD values less than 0.20. If the HIV-1 (–) Control does not meet this criteria, the entire run is invalid. Repeat the entire process (specimen and control preparation, reverse transcription, amplification and detection). If the OD values of the HIV-1 (–) Control are consistently greater than 0.20, contact the Roche Response Center (or your local Roche office, see the last page of this insert) for technical assistance.

### Positive Controls

The assigned range for the AMPLICOR HIV-1 MONITOR Low (+) and High (+) Controls **in both the Standard and UltraSensitive procedures** is specific for each lot of control and is provided on the AMPLICOR HIV-1 MONITOR Test Data Card supplied with the kit. The HIV-1 RNA copy number/mL for both the HIV-1 MONITOR Low (+) Control and the HIV-1 MONITOR High (+) Control should fall within the range indicated on the Data Card. If one or both HIV-1 MONITOR (+) Controls does not meet this criteria, the entire run is invalid. Repeat the entire process (specimen and control preparation, reverse transcription, amplification and detection). If the HIV-1 RNA copy number/mL of one or both AMPLICOR HIV-1 MONITOR (+) Controls is consistently outside the assigned range, contact the Roche Response Center (or your local Roche office, see the last page of this insert) for technical assistance.

## PROCEDURAL PRECAUTIONS

1. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Pre-Amplification Area and moving to the Post-Amplification (Amplification/Detection) Area. Pre-amplification activities must begin with reagent preparation and proceed to specimen preparation. Supplies and equipment must be dedicated to each activity and not used for other activities or moved between areas. Gloves must be worn in each area and must be changed before leaving that area. Equipment and supplies used for reagent preparation must not be used for specimen preparation activities or for pipetting or processing amplified DNA or other sources of target DNA. Post-Amplification supplies and equipment must be confined to the Post-Amplification Area at all times.

2. As with any test procedure, good laboratory technique is essential to the proper performance of this assay. Due to the high analytical sensitivity of this test and the potential for contamination, extreme care should be taken to preserve the purity of kit reagents or amplification mixtures. All reagents should be closely monitored for purity. Discard any reagents that may be suspect.

## PROCEDURE LIMITATIONS

- Monitoring the effects of antiretroviral therapy by serial measurement of plasma HIV-1 RNA has only been validated for patients with baseline viral loads ≥ 25,000 copies/mL.
- The performance of the AMPLICOR HIV-1 MONITOR Test has only been validated with HIV subtype B specimens.
- When testing specimens with viral load <200 copies/mL, the user should consider the use of well-characterized reference materials titered from 50 copies/mL to 200 copies/mL, inclusive.

1. The AMPLICOR HIV-1 MONITOR™ Test is an *in vitro* nucleic acid amplification test for the quantitation of Human Immunodeficiency Virus Type 1 (HIV-1) RNA in human plasma. The Test can quantitate HIV-1 RNA over the range of 50 - 750,000 copies/mL by using a combination of two specimen processing procedures, the Standard and UltraSensitive procedures. With the Standard procedure, the Test can accurately detect a 0.5 $\log_{10}$ (3-fold) or greater change in HIV-1 RNA if the viral load is greater than 1000 copies/mL and a 0.78 $\log_{10}$ (6-fold) or greater change when the viral load is 400 - 1000 copies/mL. With the UltraSensitive procedure, the Test can accurately detect a 0.39 $\log_{10}$ (2.5-fold) or greater change in HIV-1 RNA when the viral load is 75 - 75,000 copies/mL, a 0.44 $\log_{10}$ (2.8-fold) or greater change when the viral load is approximately 75 copies/mL, and a 0.68 log10 (5-fold) or greater change in HIV-1 RNA for patients whose viral load is approximately 50 copies/mL.

2. Reliable results are dependent on adequate specimen collection, transport, storage and processing procedures.

3. ***This test has been validated for use only with human plasma anticoagulated with EDTA or ACD. Heparin inhibits PCR; specimen collected using heparin as the anticoagulant should <u>not</u> be used with the AMPLICOR HIV-1 MONITOR Test.***

4. The presence of AmpErase® in the AMPLICOR HIV-1 MONITOR Master Mix reduces the risk of amplicon contamination. However, contamination from HIV positive controls and HIV positive clinical specimens can be avoided only by good laboratory practices and careful adherence to the procedures specified in this insert.

5. Use of this product should be limited to personnel trained in the techniques of PCR.

6. Only the PE Applied Biosystems GeneAmp PCR System 9600 or GeneAmp PCR System 2400 thermal cyclers can be used with this product.

7. As with any diagnostic test, results from the AMPLICOR HIV-1 MONITOR test should be interpreted with consideration of all clinical and laboratory findings.

## PROCEDURAL GUIDELINES

The decision of when to use the UltraSensitive specimen processing procedure will be dependent upon the laboratory's specific patient population. It is recommended that each laboratory evaluate the characteristics of the patient population (types of treatments, previous viral load results, routine or clinical trial patients) to establish the best laboratory procedure for initial testing and when to reflex to the alternate method. For example, if the patient population does not include treatment naïve patients and if all patients are known to be on antiretroviral therapy, the laboratory may elect to use the UltraSensitive specimen processing procedure as the routine method and reflex to the Standard method only when viral load results are greater than 75,000 copies/mL. If the patient population is unknown as it relates to therapy or baseline viral load results, the laboratory may elect to use the Standard specimen processing procedure as the routine method and reflex to the UltraSensitive specimen processing procedure only after the Standard specimen preparation yields a viral load below 400 copies/mL.

## INTERFERING SUBSTANCES

- ***Heparin inhibits PCR. Do not use specimens collected in heparin.***

- The following endogenous substances have been shown not to interfere with the quantitation of HIV-1-RNA by this test: triglycerides up to 1024 mg/dL, bilirubin up to 21 mg/dL and hemoglobin up to 19.5 mg/dL.

- The following drug compounds have been shown not to interfere with the quantitation of HIV-1-RNA by this test: AZT, ddI, ddC, d4T, HBY 097, nevirapine, saquinavir, isoniazid, foscarnet and ganciclovir.

## PERFORMANCE CHARACTERISTICS

### Analytical Sensitivity

The analytical sensitivity of the AMPLICOR HIV-1 MONITOR Test was established by determining the minimum number of copies of HIV-1 RNA that can be reproducibly detected by the test procedure. This was accomplished by analyzing serial dilutions of purified HIV RNA transcript. The results demonstrate that the AMPLICOR HIV-1 MONITOR Test could detect less than 2 copies of HIV RNA per reaction, and that 7.5 or more copies of HIV RNA per reaction were detected 100% of the time.

### Limit of Detection

The lower limit of detection of the AMPLICOR HIV MONITOR Test was determined by analysis of reconstructed HIV-positive plasma specimens, prepared by serial dilution of well characterized stocks of cultured HIV into HIV negative human plasma.

Standard procedure

Independent studies with blinded specimens were conducted in Europe and the United States. In the United States the performance of the Test was evaluated at RMS and in several academic laboratories with blinded specimens provided by the Virology Quality Assurance (VQA) Laboratory of the AIDS Clinical Trials Group (ACTG), Division of AIDS, National Institutes of Health. The concentration of viral RNA in the virus stocks was estimated by electron microscopy, p24 antigen, HIV-1 MONITOR Testing, and branched chain DNA analysis. The limit of detection was assessed by tabulating the number and percentage of assays for which HIV-1 RNA could not be determined

for each specimen in the panels. These studies demonstrated that the HIV-1 MONITOR Test can quantitate virion associated HIV-1 RNA in plasma at concentrations as low as 400 RNA copies/mL plasma provided that the OD of the selected microwell is within the specified OD range (0.2 to 2.0).

UltraSensitive procedure

Studies were conducted at three independent laboratories in the United States using two panels of 32 blinded specimens each, prepared using well characterized stocks of HIV spiked into HIV-negative human plasma. The concentration of viral RNA in the blinded specimens was determined prior to the study by multi-site analysis. These studies demonstrated that the AMPLICOR HIV-1 MONITOR Test can quantitate virion associated HIV-1 RNA in plasma at concentrations as low as 50 RNA copies/mL plasma provided that the OD of the selected microwell is within the specified OD range (0.2 to 2.0).

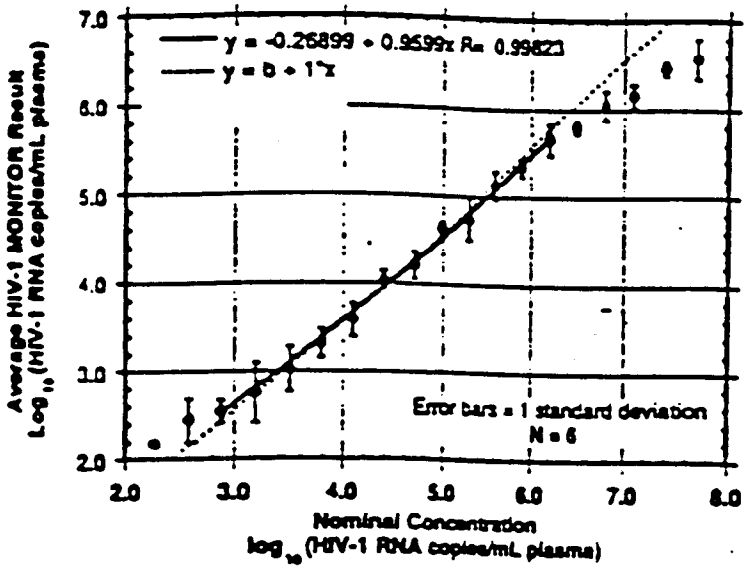
**Linear Range**

The linear range of the AMPLICOR HIV-1 MONITOR Test was determined based on the analysis of serial dilutions of well characterized stocks of cultured HIV-1 prepared in HIV-negative human plasma.

Standard procedure

An HIV-1 virus stock, characterized by electron microscopy and p24 antigen analysis, was serially diluted in HIV negative plasma and the dilutions were then tested in duplicate by three separate laboratories. The test results from the study sites were combined and then plotted on a log-log scale. As shown in Figure 3, the AMPLICOR HIV-1 MONITOR Test with Standard Specimen Processing was found to give a linear response between 400 and 750,000 HIV-1 RNA copies/mL. Specimens with results greater than 750,000 HIV-1 RNA copies/mL must be diluted with HIV-negative human plasma and retested.

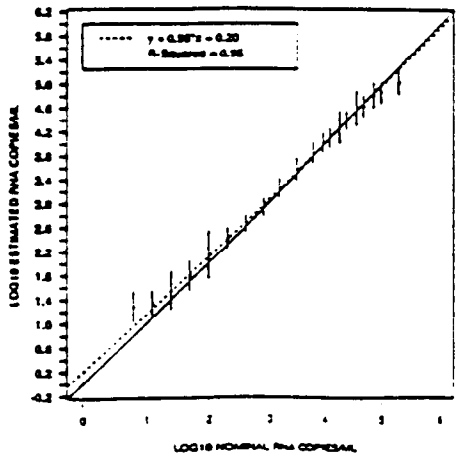
*Figure 3*
*AMPLICOR HIV-1 MONITOR Test*
*with Standard Specimen Processing*
*Linearity*


y = -0.26899 + 0.9599x R= 0.99823
y = b + 1*x
Average HIV-1 MONITOR Result
Log10 (HIV-1 RNA copies/mL plasma)
Error bars = 1 standard deviation
N = 6
Nominal Concentration
log10 (HIV-1 RNA copies/mL plasma)

UltraSensitive procedure

Serial dilutions of two well characterized viral stocks were tested in replicates of six by three separate laboratories. The test results from the study sites were combined and then plotted on a log-log scale. As shown in Figure 4, the AMPLICOR HIV-1 MONITOR Test with UltraSensitive Specimen Processing was found to give a linear response between 50 and 75,000 HIV-1 RNA copies/mL. Samples with results greater than 75,000 HIV-1 RNA copies/mL should be retested using the AMPLICOR HIV-1 MONITOR Test with Standard Specimen Processing.

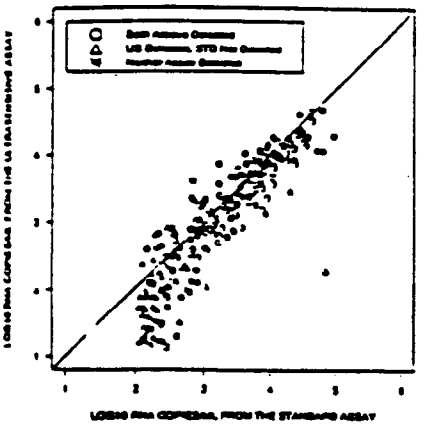
*Figure 4*
*AMPLICOR HIV-1 MONITOR Test*
*with UltraSensitive Specimen Processing*
*Linearity*



**Correlation of Results for Standard and Ultrasensitive Specimen Processing Procedures:**

In studies conducted at three separate laboratories, 310 patient specimens were tested by the AMPLICOR HIV-1 MONITOR Test using both the Standard and the UltraSensitive Specimen Processing procedures. The overall correlation for results combined across laboratories was 0.91 with a bias towards lower values (0.1 - 0.2 $\log_{10}$ lower) by the UltraSensitive procedure.

***Figure 5***
***AMPLICOR HIV-1 MONITOR Test***
***Correlation of Standard and UltraSensitive Specimen Processing Procedures***



**Analytical Specificity**

The analytical specificity of the AMPLICOR HIV-1 MONITOR Test was evaluated by adding cultured cells, cultured virus or purified nucleic acid from the following organisms and viruses to HIV negative human plasma then analyzing these spiked samples. None of the non-HIV organisms, viruses or purified nucleic acids tested showed reactivity in the AMPLICOR HIV-1 MONITOR Test. Three of the four HIV-2 isolates that were tested gave positive results. Only HIV-2 subtype A (isolate 7824A) was not detected by the AMPLICOR HIV-1 MONITOR Test; however no specific claims can be made for the ability of this Test to amplify HIV-2 isolates.

| | | |
|---|---|---|
| *Adenovirus type 2* | *Hepatitis B Virus* | *Human papilloma virus 11* |
| *Adenovirus type 3* | *Hepatitis C Virus* | *Human papilloma virus 18* |
| *Adenovirus type 7* | *HIV-2BEN* | *HTLV I/II* |
| *Cytomegalovirus (AD-169)* | *HIV-2, subtype A/B (isolate 7312A)* | *Mycobacterium avium* |
| *Cytomegalovirus Davis* | *HIV-2 subtype A (isolate 60415K)* | *Propionibacterium acnes* |
| *Epstein-Barr virus P-3* | *HIV-2 subtype A (isolate 7824A)* | *Pneumocystis carinii* |
| *Epstein-Barr virus HR1* | *Herpes simplex type I* | *Staphylococcus aureus* |
| *Epstein-Barr virus* | *Herpes simplex type II* | *Staphylococcus epidermidis* |

Limited testing has been performed on HIV-1 subtypes other than Subtype B. Preliminary experimental data in which synthetic nucleic acid constructs were used to represent the HIV-1 subtype mismatches with primers SK462 and SK431 indicate that non-B subtypes of HIV-1 will be amplified by the AMPLICOR HIV-1 MONITOR Test with reduced efficiencies. The reduced amplification efficiency depends upon the number of mismatches with the SK462 and SK431 primers. Group O specimens will not be amplified by this test.

## Clinical Specificity

The clinical specificity of the AMPLICOR HIV-1 MONITOR Test was determined by analysis of 495 anti-HIV-1 negative blood donors. None of these specimens was reactive with the AMPLICOR HIV-1 MONITOR Test. Assuming a zero prevalence of HIV-1 infection in the seronegative blood donors, the specificity of the test was 100%.

***Anti-HIV-1 Negative Blood Donors***

| N | HIV RNA copies/mL | | HIV $OD_{450}$ (in row A of the Microwell Plate) | | | | |
|---|---|---|---|---|---|---|---|
| | Not Detected | Positive | Minimum | Average | Maximum | SD | % CV |
| 495 | 495 | 0 | 0.033 | 0.053 | 0.130 | 0.012 | 22.6 |

The AMPLICOR HIV-1 MONITOR Test will amplify HIV-1 DNA if it is present in the processed specimen. In-house testing using a modification of the AMPLICOR HIV-1 MONITOR Test which made the test specific and more sensitive for the qualitative detection of HIV-1 DNA was performed on fifty-five HIV positive plasma specimens. This testing showed that fourteen specimens had plasma DNA present. Five of the fourteen specimens that were shown to contain plasma DNA were retested by the AMPLICOR HIV-1 MONITOR Test but without the reverse transcription step so that only plasma DNA would be amplified. The results from these tests showed that DNA was undetectable by the AMPLICOR HIV-1 MONITOR Test in four of the specimens and contributed to less than 10% of the apparent HIV-1 RNA test result in the fifth specimen.

## Precision

Within-Run and Total Precision were evaluated according to the methods defined in the NCCLS Guideline (EP5-T2), "Evaluation of Precision Performance of Clinical Chemistry Devices". This procedure permits the determination of both Within-Run and Total Precision through the performance of a multiple day and multiple operator study.

Standard procedure

The precision of the AMPLICOR HIV-1 MONITOR Test with Standard Specimen Processing was evaluated using 6 specimens at different HIV-1 RNA concentrations. The study was performed in the Roche Molecular Systems Diagnostic Development laboratories. A run consisting of 24 tests was performed daily for 10 days by each of 2 operators. Since only 12 specimens can be run on a single HIV-1 MONITOR MWP, this required each operator to run the test on 2 separate microwell plates each day. The order of specimens in each run was changed each day. Each run included 4 replicates of each of 6 specimens. The six specimens were taken through the entire HIV-1 MONITOR Test procedure, including sample preparation using the Standard Specimen Preparation procedure, reverse transcription, PCR amplification and detection. Therefore, the precision reported here takes into account all aspects of the test procedure. The calculation of Within-Run and Total Precision was performed according to the methods defined in the EP5-T2 Guideline. The results from this study are shown in Table 2a.

UltraSensitive Procedure

The precision of the AMPLICOR HIV-1 MONITOR Test with UltraSensitive Specimen Processing was evaluated by testing 6 specimens prepared by spiking basematrix plasma with HIV-1 virus stock to the following concentrations (75, 500, 2500, 25,000 and 50,000 copies/mL). Testing was performed at 3 different laboratories. All specimens were tested in replicates of 4 on each of 10 days at each laboratory. The 6 specimens were taken through the entire HIV-1 MONITOR Test procedure, including sample preparation using the UltraSensitive Specimen Preparation procedure, reverse transcription, PCR amplification and detection. Therefore, the precision reported here takes into account all aspects of the test procedure. The calculation of Within-Run and Total Precision was performed according to the methods defined in the EP5-T2 Guideline. The results from this study are shown in Table 2b.

***Table 2a***
***Precision of the AMPLICOR HIV-1 MONITOR Test with Standard Specimen Processing***

| Sample | (–) Control | Low (+) Control* | High (+) Control* | 1† | 2† | 3† |
|---|---|---|---|---|---|---|
| Total replicates | 80 | 80 | 80 | 80 | 80 | 80 |
| Mean copies/mL | none | 2,043 | 768,767 | 2,108 | 39,386 | 1,156,875 |
| **WITHIN-RUN** | | | | | | |
| Standard Deviation | - | 4 | 229,499 | 2,071 | 12,937 | 398,522 |
| CV (%) | - | 30.8 | 29.8 | 98.2 | 32.8 | 34.4 |
| **TOTAL** | | | | | | |
| Standard Deviation | - | 660 | 325,561 | 2,209 | 16,169 | 524,528 |
| CV (%) | - | 32.3 | 42.3 | 104.8** | 41.1 | 45.3 |

***Table 2b***
***Precision of the AMPLICOR HIV-1 MONITOR Test with UltraSensitive Specimen Processing***

| Sample | 1† | 2† | 3† | 4† | 5† |
|---|---|---|---|---|---|
| Nominal Copies/mL | 75 | 500 | 2,500 | 25,000 | 50,000 |
| Mean copies/mL | 86.9 | 536 | 2,425 | 21,715 | 41,282 |
| Total replicates | 120 | 120 | 120 | 120 | 120 |
| **WITHIN-RUN** | | | | | |
| Standard Deviation | 27.4 | 120 | 502 | 6,130 | 11,846 |
| CV (%) | 31.6 | 22.5 | 20.7 | 28.2 | 28.7 |
| **TOTAL** | | | | | |
| Standard Deviation | 29.1 | 162 | 623 | 8,902 | 17,313 |
| CV (%) | 33.5 | 30.2 | 25.7 | 41.0 | 41.9 |

* RNA Transcript
† - Dilutions of viral stocks in human plasma

**Reproducibility**

Assay reproducibility was determined by assessing intra-assay, inter-assay and inter-lab variability.

Standard procedure

Reproducibility data were generated by testing four panels of specimens at multiple laboratories over a one year period. The study was performed by 49 laboratories participating in the AIDS Clinical Trial Group (ACTG) Virology Quality Assurance (VQA) Program. In total, the panels included 22 separate specimens (9 patient specimens and 13 spiked plasma specimens). The patient specimens ranged in concentration from 500 to 364,000 copies/mL; the spiked specimens ranged in concentration from 500 to 275,000 copies/mL. A total of 2,294 test results were generated for the specimens from the four panels.

The analysis of assay variability included separate assessments of Intra-Assay, Inter-Assay, Inter-Laboratory and Total variance for specimens with HIV-1 RNA viral loads ≥ 1000 copies/mL and for specimens with HIV-1 RNA viral loads of approximately 500 copies/mL. The results from the analysis of the data for specimens with HIV-1 RNA levels of approximately 500 copies/mL and ≥1000 copies/mL are shown in Table 3. The analyses were performed after the test results were log10 transformed.

To assess assay reproducibility at approximately 500 copies/mL and at ≥ 1000 copies/mL level, the standard deviation of the sum of the two important sources of assay variance (Intra-Assay and Inter-Assay) was determined and the 95% confidence interval for the standard deviation was calculated. Table 4 contains the results of these calculations. These data show that the AMPLICOR HIV-1 MONITOR™ Test can accurately detect a 0.5 $\log_{10}$ (3-fold) or greater change in HIV-1 RNA for patients whose viral load is ≥ 1000 copies/mL and accurately detect a 0.78 $\log_{10}$ (6-fold) or greater change in HIV-1 RNA for patients whose viral load is below 1000 copies/mL.

UltraSensitive procedure

Reproducibility data were generated by testing 5 samples at three laboratories over a period of 10 days. The samples were prepared by dilution of viral stocks in HIV-negative human plasma and ranged in concentration from 75 to 50,000 copies/mL. The analysis of assay variability included separate assessments of Intra-Assay, Inter-Assay, Inter-Laboratory and Total variance for specimens with HIV-1 RNA viral loads ≥ 75 copies/mL and for specimens with HIV-1 RNA viral loads of approximately 75 copies/mL and viral loads of approximately 50 copies/mL. The results from the analysis of these specimens are shown in Table 3. The analyses were performed after the test results were log10 transformed.

To assess assay reproducibility at approximately 50 copies/mL, 75 copies/mL and ≥ 75 copies/mL, the standard deviation of the sum of the two important sources of assay variance (Intra-Assay and Inter-Assay) was determined and the 95% confidence interval for the standard deviation was calculated. Table 4 contains the results of these calculations. These data show that the AMPLICOR HIV-1 MONITOR™ Test with UltraSensitive Specimen Processing can accurately detect a 0.39 $\log_{10}$ (2.5-fold) or greater change in HIV-1 RNA for patients whose viral load is ≥ 75 copies/mL and a 0.44 $\log_{10}$ (2.8-fold) or greater change in HIV-1 RNA for patients whose viral load is approximately 75 copies/mL, and a 0.68 log10 (5-fold) or greater change in HIV-1 RNA for patients whose viral load is approximately 50 copies/mL.

***Table 3***
***Components of Variance - AMPLICOR HIV-1 MONITOR Test:***
***Log10 Estimated RNA***

| RNA Level | # Labs | Variance Component | Standard Deviation | Variance | % of Total Variance |
|---|---|---|---|---|---|
| 50 copies/mL (UltraSensitive Procedure) | 3 | Total | 0.2482 | 0.0616 | 100 |
| | | Inter-Lab | 0.0316 | 0.0010 | 1.6 |
| | | Inter-Assay | 0 | 0 | 0 |
| | | Intra-Assay | 0.2462 | 0.0606 | 98.4 |
| 75 copies/mL (UltraSensitive Procedure) | 3 | Total | 0.2045 | 0.0422 | 100 |
| | | Inter-Lab | 0.1288 | 0.0166 | 39.3 |
| | | Inter-Assay | 0.0265 | 0.0007 | 1.7 |
| | | Intra-Assay | 0.1579 | 0.0249 | 59.0 |
| ≥75 copies/mL (UltraSensitive Procedure) | 3 | Total | 0.1661 | 0.0276 | 100 |
| | | Inter-Lab | 0.0906 | 0.0082 | 29.8 |
| | | Inter-Assay | 0.0814 | 0.0066 | 24.0 |
| | | Intra-Assay | 0.1130 | 0.0128 | 46.2 |
| 500 copies/mL (Standard Procedure) | 18 | Total | 0.2966 | 0.0880 | 100 |
| | | Inter-Lab | 0.1131 | 0.0128 | 14.5 |
| | | Inter-Assay | 0.0470 | 0.0022 | 2.5 |
| | | Intra-Assay | 0.2702 | 0.0730 | 83.0 |
| ≥1000 copies/mL (Standard Procedure) | 29 | Total | 0.1861 | 0.0346 | 100 |
| | | Inter-Lab | 0.0453 | 0.0021 | 5.9 |
| | | Inter-Assay | 0.1021 | 0.0104 | 30.1 |
| | | Intra-Assay | 0.1489 | 0.0222 | 64.0 |

***Table 4***
***Assay Reproducibility - AMPLICOR HIV-1 MONITOR Test***

| RNA Level | Σ of Variance* | SD of Variance | 95% CI |
|---|---|---|---|
| 50 copies/mL (UltraSensitive Procedure) | 0.0606 | 0.3481 | ± 0.68 log10 |
| 75 copies/mL (UltraSensitive Procedure) | 0.0256 | 0.2263 | ± 0.44 log10 |
| ≥ 75 copies/mL (UltraSensitive Procedure) | 0.0194 | 0.1970 | ± 0.39 log10 |
| 500 copies/mL (Standard Procedure) | 0.0752 | 0.3878 | ± 0.76 log10 |
| ≥ 1000 copies/mL (Standard Procedure) | 0.0326 | 0.2553 | ± 0.50 log10 |

* for Inter-Assay and Intra-Assay Variance

**Clinical Performance**

*Patient Prognosis*

The use of the AMPLICOR HIV-1 MONITOR Test to predict the risk of disease progression in HIV infected individuals was evaluated in ACTG studies 116A and 116B/117. The data from these studies were analyzed by the Cox Proportional Hazards Model to evaluate the frequency of disease progression based upon HIV-1 RNA level. ACTG Study 116A was a double-blinded study that compared the clinical efficacy of zidovudine (ZDV) in combination with two doses of 2',3'-dideoxyinosine (ddI) in patients with advanced HIV disease who had up to 16 weeks of prior treatment with zidovudine. ACTG Study 116B/117 was an efficacy study which compared 2',3'-dideoxyinosine (ddI) and zidovudine therapy of patients with HIV infection who had been on zidovudine treatment for more than 16 weeks. The patient population in each of these studies included patients with a diagnosis of AIDS at study baseline, ARC at baseline and asymptomatic patients at baseline. Disease progression was defined as progression to AIDS, new AIDS defining event, or death.

The ACTG 116A HIV-1 RNA sub-study included 186 patients from the clinical study who had had plasma and PBMC specimens collected upon entrance into the study and whose specimens had been stored appropriately and were available for testing in the sub-study. The ACTG 116B/117 HIV-1 RNA sub-study included 99 randomly selected patients from the drug study who had plasma specimens collected on entrance into the study and whose specimens had been stored appropriately and were available for testing in the sub-study.

The unadjusted and adjusted relative hazards for disease progression as measured by baseline HIV-1 RNA levels, change in HIV-1 RNA levels over 8 weeks, and CD4+ cell counts were evaluated using Cox Proportional Hazards Models. The unadjusted Relative Hazard represents the risk conferred by the variable alone, whereas the Adjusted Relative Hazard represents the risk conferred by that variable after controlling for the other variables in the model. These models give the increased risk (if any) of disease progression associated with the variables entered into the model. The analyses were performed by assessing the relative hazards of a 5-fold difference in the study variable. The results of these analyses are presented in Tables 5 through 8. These data show that in a population of patients with advanced HIV disease and undergoing specific anti-reverse transcriptase therapies, 5-fold higher baseline HIV-1 RNA levels are associated with increased risk of disease progression. For patients who have had greater than 16 weeks of prior ZDV therapy (patients in ACTG Study 116B/117), 5-fold higher baseline HIV-1 RNA levels have not proven to be of significant prognostic value. For patients who have had either no prior ZDV therapy or up to 16 weeks or less of ZDV therapy, 5-fold changes between baseline and Week 8 RNA levels have significant prognostic value. For patients who have had greater than 16 weeks of prior ZDV therapy, 5-fold changes between baseline and Week 8 RNA levels have not proven to be of significant prognostic value.

The frequency of disease progression was also analyzed for each study by dividing each study population into deciles by rank order of baseline HIV-1 RNA. The deciles were evaluated for the frequency of disease progression. For each study, a frequency of disease progression of $\geq$ 60% was found for all patients with baseline HIV-1 RNA levels above 250,000 copies/mL. In Study 116A, an approximate 35% frequency of disease progression was found for patients in the first four deciles (<11912, <34661, <72438 and <103806 HIV-1 RNA copies/mL). The frequency of disease progression was between 40% and 50% in the next three deciles (<15695, <194312 and <247229). In the last three deciles, where the HIV-1 RNA levels were >250000 HIV-1 RNA copies/mL, the frequency of disease progression was greater than 60%. In Study 116B/117 the frequency of disease progression was found to be more variable, but still showed the general trend of higher

rates of progression with increased HIV-1 RNA levels. An average 30% disease progression rate (range = 10% - 60%) was found for the first six deciles in this study (<11571, <31292 and <49743, <62132, <97781 and <150866). The rate of disease progression increased to 50% and 60% for the next two deciles (<251627 and <403146, respectively). The disease progression rates were greater than 80% for the last two deciles (<794027 and <1456302) where the HIV-1 RNA levels were greater than 403,000. Summary tables and bar charts of these analyses are presented in Figures 4 and 5.

*Table 5*
*Association of Study Variables at Baseline with Disease Progression*
*ACTG Study 116A*
*(N = 153 Patients, 73 Progression Events)*

| Variable | Unadjusted Relative Hazard (95% CI) | Adjusted Relative Hazard (95% CI) | p value[2] |
|---|---|---|---|
| Log HIV-1 RNA copy number[1] | 1.58 (1.20 - 2.09) | 1.44 (1.07 -1.93) | 0.02 |
| Log CD4+ cell count[1] | 0.39 (0.28 - 0.54) | 0.45 (0.31 -0.64) | 0.0001 |
| Dx of AIDS at Baseline | 2.00 (1.22 - 3.27) | 1.39 (0.82 - 2.37) | 0.22 |
| ddI Treatment | 0.95 (0.59 - 1.53) | 1.12 (0.68 - 1.84) | 0.66 |

1 - The Relative Hazard is the hazard ratio resulting from a 5-fold increase in HIV-1-RNA or decrease in CD4 cell count
2 - p values are for Adjusted Relative Hazards

*Table 6*
*Association of Change in HIV-1-1 RNA from Baseline to Week 8 with Disease Progression*
*ACTG Study 116A*
*(N = 114 Patients, 62 Progression Events)*

| Variable | Unadjusted Relative Hazard (95% CI) | Adjusted Relative Hazard (95% CI) | p value[2] |
|---|---|---|---|
| Log HIV-1 RNA copy number[1] | 1.46 (1.11 - 1.93) | 1.63 (1.16 -2.28) | 0.0005 |
| Log Change in HIV-1-1 RNA from Baseline to Week 8[1] | 1.18 (0.93 - 1.48) | 1.54 (1.09 - 2.16) | 0.013 |
| Log CD4+ cell count[1] | 0.43 (0.30 - 0.62) | 0.50 (0.34 -0.73) | 0.0004 |
| Dx of AIDS at Baseline | 1.83 (1.09 - 3.09) | 1.28 (0.74 - 2.21) | 0.38 |
| ddI Treatment | 0.76 (0.45 - 1.27) | 0.87 (0.51 - 1.49) | 0.61 |

1 - The Relative Hazard is the hazard ratio resulting from a 5-fold increase in HIV-1-RNA or decrease in CD4 cell count
2 - p values are for Adjusted Relative Hazards

*Table 7*
*Association of Study Variables at Baseline with Disease Progression*
*ACTG Study 116B/117*
*(N = 86 Patients, 39 Progression Events)*

| Variable | Unadjusted Relative Hazard (95% CI) | Adjusted Relative Hazard (95% CI) | p value[2] |
|---|---|---|---|
| Log HIV-1 RNA copy number[1] | 1.90 (1.28 - 2.82) | 1.25 (0.81 -1.94) | 0.32 |
| Log CD4+ cell count[1] | 0.28 (0.16 - 0.48) | 0.33 (0.18 -0.62) | 0.0006 |
| Dx of AIDS at Baseline | 3.13 (1.66 - 5.92) | 2.38 (1.24 - 4.58) | 0.01 |
| ddI Treatment | 0.89 (0.47 - 1.69) | 0.88 (0.46 - 1.71) | 0.71 |

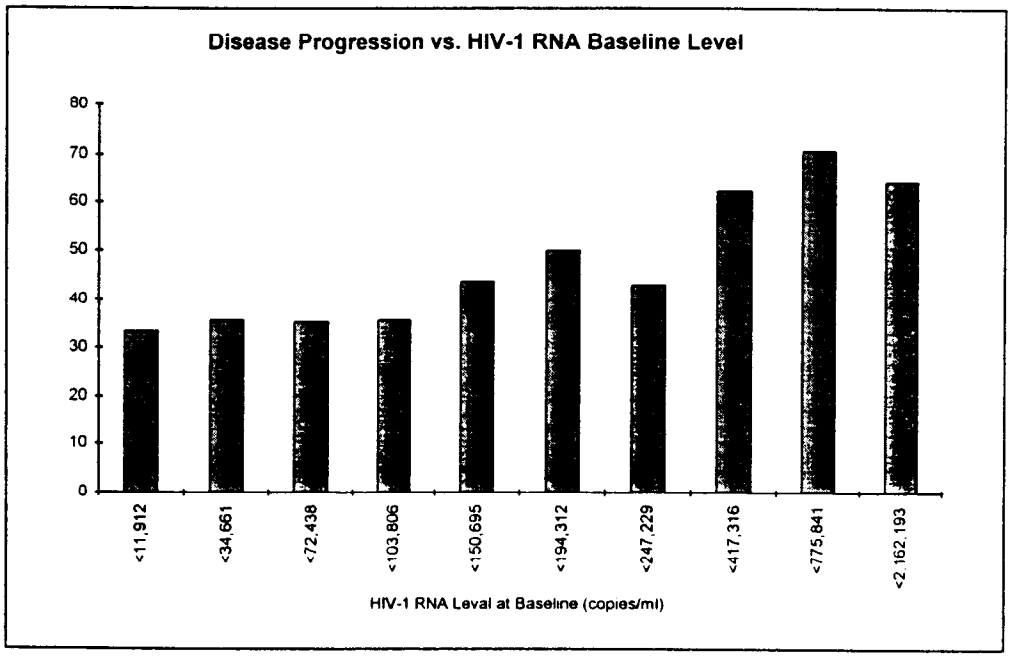
1 - The Relative Hazard is the hazard ratio resulting from a 5-fold increase in HIV-1-RNA or decrease in CD4 cell count
2 - p values are for Adjusted Relative Hazards

*Table 8*
*Association of Change in HIV-1-1 RNA from Baseline to Week 8 with Disease Progression*
*ACTG Study 116B/117*
*(N = 65 Patients, 29 Progression Events)*

| Variable | Unadjusted Relative Hazard (95% CI) | Adjusted Relative Hazard (95% CI) | p value[2] |
|---|---|---|---|
| Log HIV-1 RNA copy number[1] | 2.10 (1.32 - 3.34) | 1.58 (0.93 -2.69) | 0.09 |
| Log Change in HIV-1-1 RNA from Baseline to Week 8[1] | 1.41 (0.71 - 2.79) | 1.58 (0.68 - 3.68) | 0.29 |
| Log CD4+ cell count[1] | 0.25 (0.13 - 0.46) | 0.29 (0.14 -0.60) | 0.001 |
| Dx of AIDS at Baseline | 2.43 (1.17 - 5.05) | 1.87 (0.88 - 3.97) | 0.10 |
| ddI Treatment | 1.07 (0.51 - 2.27) | 0.98 (0.39 - 2.48) | 0.96 |

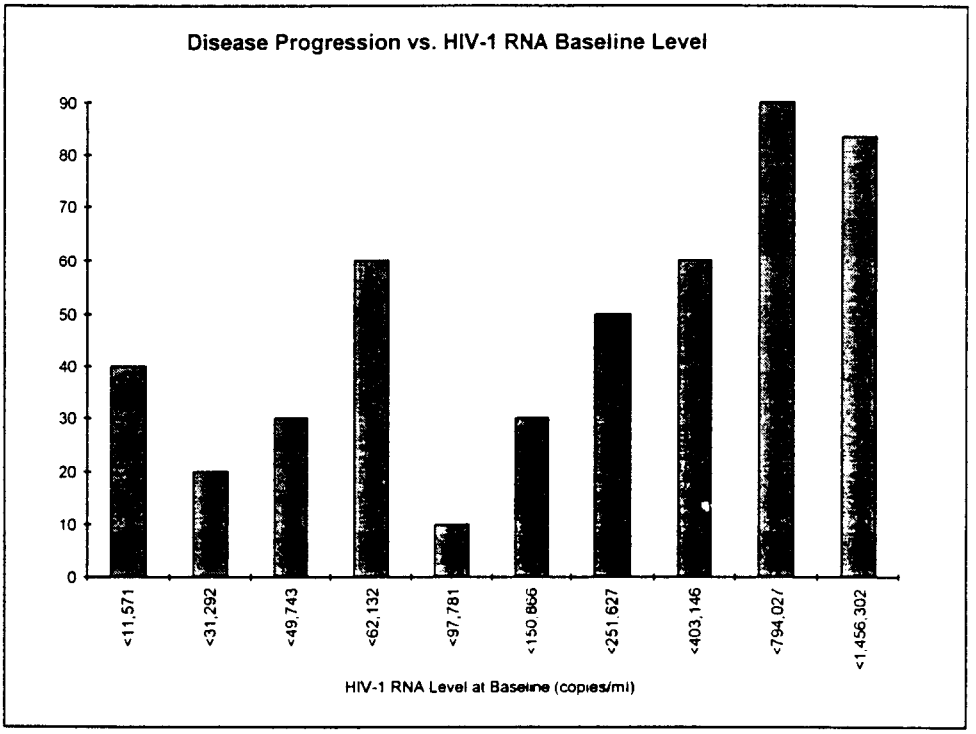
1 - The Relative Hazard is the hazard ratio resulting from a 5-fold increase in HIV-1-RNA or decrease in CD4 cell count
2 - p values are for Adjusted Relative Hazards

*Figure 4*
*Frequency of Disease Progression by Baseline HIV-1 RNA level*
*Study 116A*




| Record # | Decile | n | # Progressions | # Progressing to AIDS/Death | % |
|---|---|---|---|---|---|
| 18 | <11912 | 18 | 15 | 5 | 33.33 |
| 36 | <34661 | 18 | 14 | 5 | 35.71 |
| 54 | <72438 | 18 | 17 | 6 | 35.29 |
| 72 | <103806 | 18 | 14 | 5 | 35.71 |
| 90 | <150695 | 18 | 16 | 7 | 43.75 |
| 108 | <194312 | 18 | 16 | 8 | 50.00 |
| 126 | <247229 | 18 | 14 | 6 | 42.86 |
| 144 | <417316 | 18 | 16 | 10 | 62.50 |
| 162 | <775841 | 18 | 17 | 12 | 70.59 |
| 179 | <2162193 | 17 | 14 | 9 | 64.29 |

*Figure 5*
*Frequency of Disease Progression by Baseline HIV-1 RNA level*
*Study 116B/117*




| Record # | Decile | n | # Progressions | # Progressing to AIDS/Death | % |
|---|---|---|---|---|---|
| 10 | <11571 | 10 | 10 | 4 | 40.00 |
| 20 | <31292 | 10 | 10 | 2 | 20.00 |
| 30 | <49743 | 10 | 10 | 3 | 30.00 |
| 40 | <62132 | 10 | 10 | 6 | 60.00 |
| 50 | <97781 | 10 | 10 | 1 | 10.00 |
| 60 | <150866 | 10 | 10 | 3 | 30.00 |
| 70 | <251627 | 10 | 10 | 5 | 50.00 |
| 80 | <403146 | 10 | 10 | 6 | 60.00 |
| 90 | <794027 | 10 | 10 | 9 | 90.00 |
| 96 | <1456302 | 6 | 6 | 5 | 83.33 |

*Measuring Response to Antiretroviral Therapy*

The use of the AMPLICOR HIV-1 MONITOR Test to measure the effects of antiretroviral therapy was evaluated in a clinical study of antiretroviral compounds including the reverse transcriptase inhibitor zalcitabine (ddC, tradename HIVID®), the protease inhibitor drug Saquinavir (SAQ, trade name INVIRASE®), and combinations of these two drugs. The study (Study NV14256) was a double–blind, phase III randomized study whose primary objective was to evaluate the safety, tolerability and efficacy of three treatments (ddC, SAQ, and SAQ in combination with ddC) based on clinical endpoints in patients discontinuing (or unable to take) zidovudine (ZDV) therapy and to compare survival among the three treatment groups (including death after an AIDS Defining Event (ADE), or dose limiting toxicity).

For Study NV14256, prior ZDV treatment was an inclusion criterion with the majority of patients having over one year of prior ZDV treatment. A total of 970 patients were entered into Study NV14256 at forty nine clinical sites. The study had the following three treatment arms (patients): ddC (325), Saquinavir (327), and Saquinavir + ddC (318). The demographic and baseline disease characteristics of the patients in Study NV14256 represented a diverse population of patients with advanced HIV-1 infection and with a wide range of prior antiretroviral treatment regimes.

The utility of the AMPLICOR HIV-1 MONITOR Test to measure the effects of drug treatment over time was evaluated by analyzing the median change from baseline and the DAVGt (difference averaged over time - mean change from baseline over t weeks) in the HIV-1 RNA level. To evaluate the ability of the AMPLICOR HIV-1 MONITOR Test to detect changes in HIV-1 RNA levels as a result of therapy, the median change from baseline was analyzed over time for each treatment arm of the study. Figure 6 shows the median change from baseline over a 48 week time period for each treatment arm in Study NV14256. Measurable and sustained decreases in HIV-1 RNA levels as determined by the AMPLICOR HIV-1 MONITOR Test were seen. The largest and the most sustained median change in HIV-1 RNA levels at each time point was seen in patients in the combination treatment arm (SAQ+ddC).

The utility of serial HIV-1 RNA measurements to assess viral response to antiretroviral treatment was also examined using the Cox Proportional Hazards Model. In this analysis, the three treatment groups were combined and a Cox model, stratified by treatment group, was fit using the following covariates as linear terms: log10(baseline HIV-1 RNA), log10(last HIV-1 RNA), baseline CD4 and last CD4. The hazard ratio in this model was defined as a 10-fold increase in HIV-1 RNA or a decrease of 100 in CD4 count. As shown in Table 9, the coefficient of the log10(last HIV-1 RNA) term for this model is statistically significant and positive through Week 40. Accordingly, the HIV-1 RNA Hazard Ratios through Week 40 indicate that the HIV-1 RNA levels at each time point have statistically significant and continuous prognostic value.

In a related analysis of the data, the association between HIV-1 RNA levels and survival time was assessed. For this analysis, survival time was defined as time on study in which a patient survived free of an AIDS Defining Event (ADE) or death. The analyses were performed by constructing Kaplan-Meier Survival Curves for each treatment arm by dividing the patient population within the treatment arm into thirds based on HIV-1 RNA levels, nominally the Lower, Middle, and Upper thirds. The survival curves were plotted as a function of the percent ADE Survival for each patient group (Lower, Middle and Upper) for the number of weeks following Week 8. The Kaplan Meier estimated survival analyses show that patients with low HIV-1 RNA levels (Lower Third) are more likely to survive ADE-free for a longer period of time than patients with high HIV-1 RNA levels. The Kaplan-Meier survival curves for each study treatment arm are shown in Figures 7, 8 and 9.

In a separate analysis of the data from Study NV14256, a patient's risk of ADE or death was assessed as a function of the last (most recent) HIV-1 RNA level at various study weeks. In particular, the impact of the last HIV-1 RNA measurement on the hazard for the development of a first ADE or death (based upon clinical events seen later in the study) was assessed. The analysis was performed using a Cox model survival analysis using the covariates log10(baseline HIV-1 RNA), log10(last HIV-1 RNA), baseline CD4 and last CD4. The model was a linear function of the covariates predicting the log of the hazard (ratio) for an ADE. The linear function was then used to determine a Risk Index for all patients in the study. Patients who survived ADE free through Week 4 of the study were sorted by their Risk Index with the first 25% of the patients forming the "low risk" group and the remaining patients equally divided into six separate groups according to their Risk Index. Each of these six groups was then compared to the low risk group using a survival model and the mean HIV-1 RNA level of each group was plotted versus an ADE hazard ratio calculated using a piecewise exponential model. Figures 10 and 11 show the Hazard Ratios for the time to first ADE versus the log10(last HIV-1 RNA) for patients surviving ADE free through Week 4 and patients surviving ADE free through Week 16, respectively. These data show that a patient in this study at Week 4 with an HIV-1 RNA level of 100,000 copies/mL (log10 =5.0) was 6 times more likely to develop an ADE or to die than was patient with an HIV-1 RNA level of 8000 copies/mL. A patient in this study at Week 16 with an HIV-1 RNA level of 1,000,000 (log10 = 6.0) was 20 times more likely to develop an ADE or to die than was a patient with an HIV-1 RNA level of 8,000 copies/mL.

The data presented here regarding the clinical utility and the use of the AMPLICOR HIV-1 MONITOR Test to monitor the effects of antiretroviral therapy were derived from a single clinical study that compared two drugs and three therapeutic regimens for a specific patient group. Because a single clinical study may not adequately demonstrate the clinical utility of quantitative HIV RNA testing in all patient populations, in all clinical situations, or with all antiretroviral therapies, sufficient care should be taken before extending the interpretation of the data from the study presented here to any individual patient case. As with any diagnostic test, results from the AMPLICOR HIV-1 MONITOR Test should be interpreted with consideration of all relevant clinical and laboratory findings for each patient.

*Figure 6*
*HIV-1 RNA Medians of Change from Baseline Over 48 Weeks*
*Study NV14256*

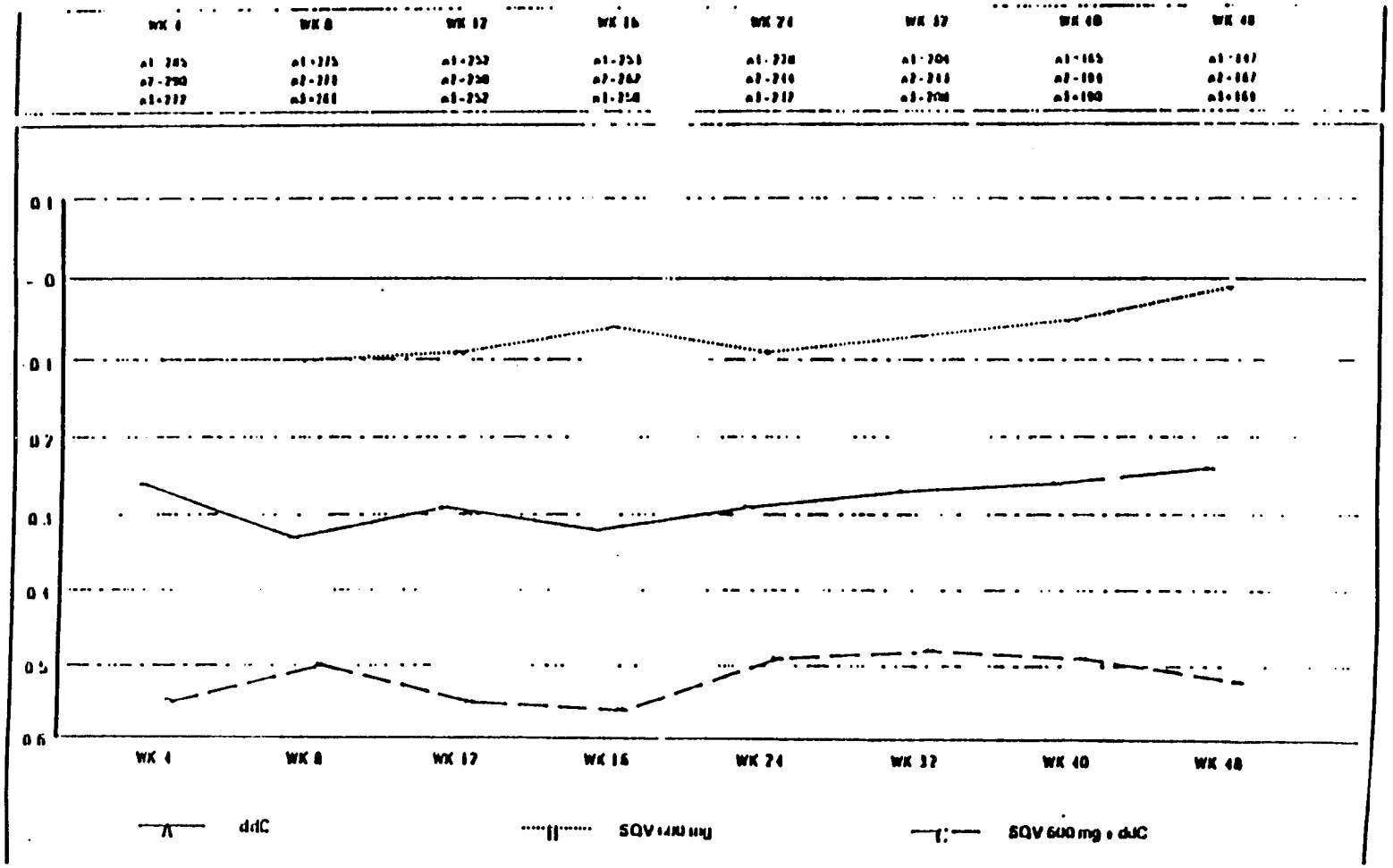


*Table 9*
*ADE Risk versus Log10(Last RNA) and Other Covariates*
*Based on Time from the Given Study Week for Patients Surviving Through that Week*
*Summary of Cox Model Results - Model Stratified by Treatment Group*
*Study NV14256*

| Study Week | Variable | Cox Model Coefficient | Hazard Ratio* | p Value |
|---|---|---|---|---|
| | log10(Baseline RNA) | 0.798 | 2.22 | 0.0012 |
| 4 | log10(Last RNA) | 0.4197 | 1.52 | 0.0219 |
| | Baseline CD4 | -0.0009 | 0.91 | 0.5806 |
| | Last CD4 | -0.0040 | 0.67 | 0.0084 |
| | log10(Baseline RNA) | 0.8096 | 2.25 | 0.0004 |
| 8 | log10(Last RNA) | 0.4186 | 1.52 | 0.0138 |
| | Baseline CD4 | -0.0019 | 0.83 | 0.2107 |
| | Last CD4 | -0.0049 | 0.62 | 0.0006 |
| | log10(Baseline RNA) | 0.8714 | 2.39 | 0.005 |
| 16 | log10(Last RNA) | 0.5385 | 1.71 | 0.0046 |
| | Baseline CD4 | -0.0015 | 0.87 | 0.3510 |
| | Last CD4 | -0.0049 | 0.62 | 0.0010 |
| | log10(Baseline RNA) | 0.6910 | 2.00 | 0.0136 |
| 24 | log10(Last RNA) | 0.7135 | 2.04 | 0.0017 |
| | Baseline CD4 | -0.0003 | 0.97 | 0.8667 |
| | Last CD4 | -0.0064 | 0.53 | 0.0001 |
| | log10(Baseline RNA) | 0.5926 | 1.81 | 0.0492 |
| 32 | log10(Last RNA) | 0.9000 | 2.46 | 0.0003 |
| | Baseline CD4 | -0.0040 | 0.67 | 0.0252 |
| | Last CD4 | -0.0023 | 0.79 | 0.1523 |
| | log10(Baseline RNA) | 0.8003 | 2.23 | 0.0159 |
| 40 | log10(Last RNA) | 0.7086 | 2.03 | 0.0096 |
| | Baseline CD4 | -0.0030 | 0.74 | 0.1282 |
| | Last CD4 | -0.0031 | 0.73 | 0.0955 |
| | log10(Baseline RNA) | 0.5993 | 1.82 | 0.0978 |
| 48 | log10(Last RNA) | 0.5631 | 1.76 | 0.0629 |
| | Baseline CD4 | -0.0011 | 0.89 | 0.6168 |
| | Last CD4 | -0.0079 | 0.45 | 0.0016 |
| | log10(Baseline RNA) | 1.0917 | 2.98 | 0.0127 |
| 56 | log10(Last RNA) | 0.0835 | 1.09 | 0.8025 |
| | Baseline CD4 | -0.0022 | 0.80 | 0.4560 |
| | Last CD4 | -0.0072 | 0.49 | 0.0201 |
| | log10(Baseline RNA) | 1.2803 | 3.60 | 0.0135 |
| 64 | log10(Last RNA) | -0.2500 | 0.78 | 0.4988 |
| | Baseline CD4 | 0.0006 | 1.07 | 0.8531 |
| | Last CD4 | -0.0085 | 0.43 | 0.0213 |

* Hazard Ratio due to a 10 fold increase in HIV-1 RNA or a decrease of 100 in CD4 count

Figure 7
Kaplan Meier Estimated Survivals
Study NV14256 Treatment Group: SAQ 600mg
Patients Surviving ADE Free Through Week 8

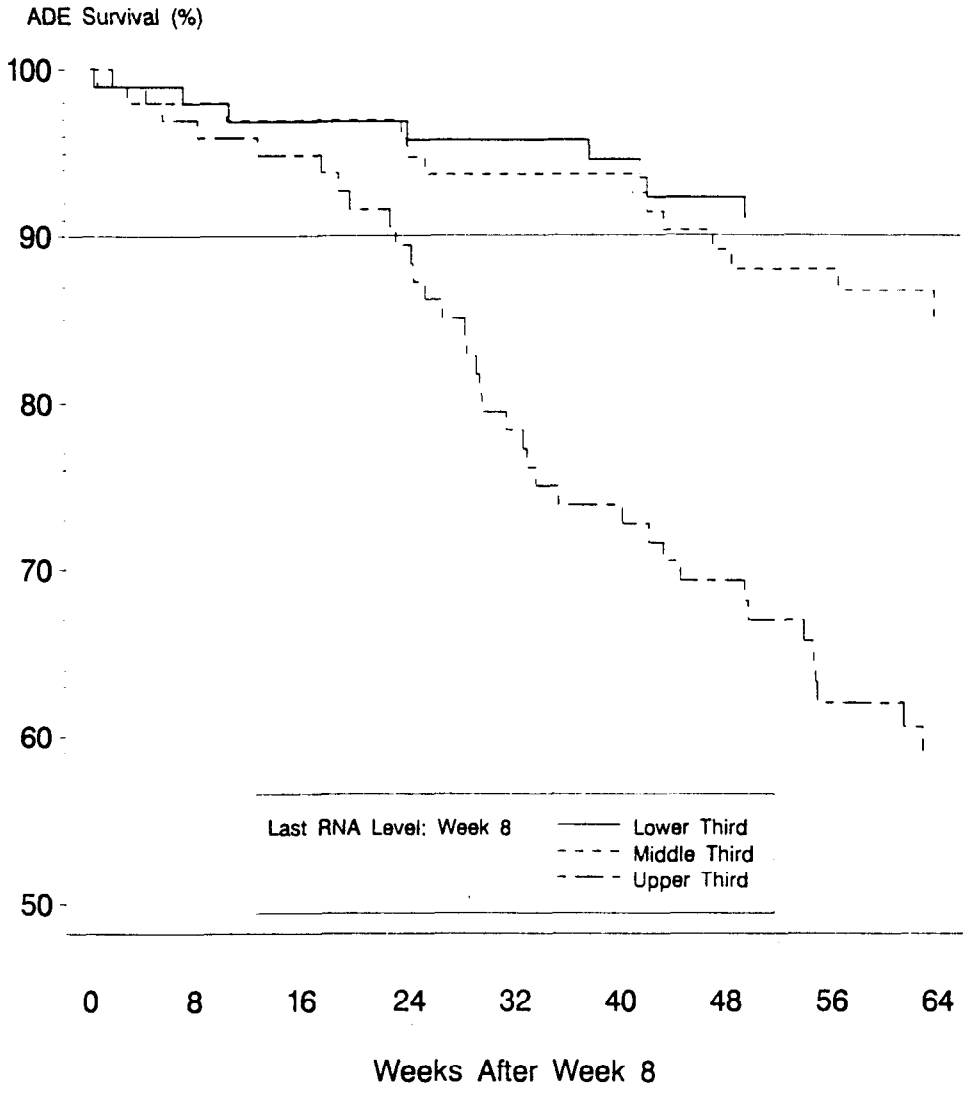


Figure 8
Kaplan Meier Estimated Survivals
Study NV14256 Treatment Group: ddC
Patients Surviving ADE Free Through Week 8

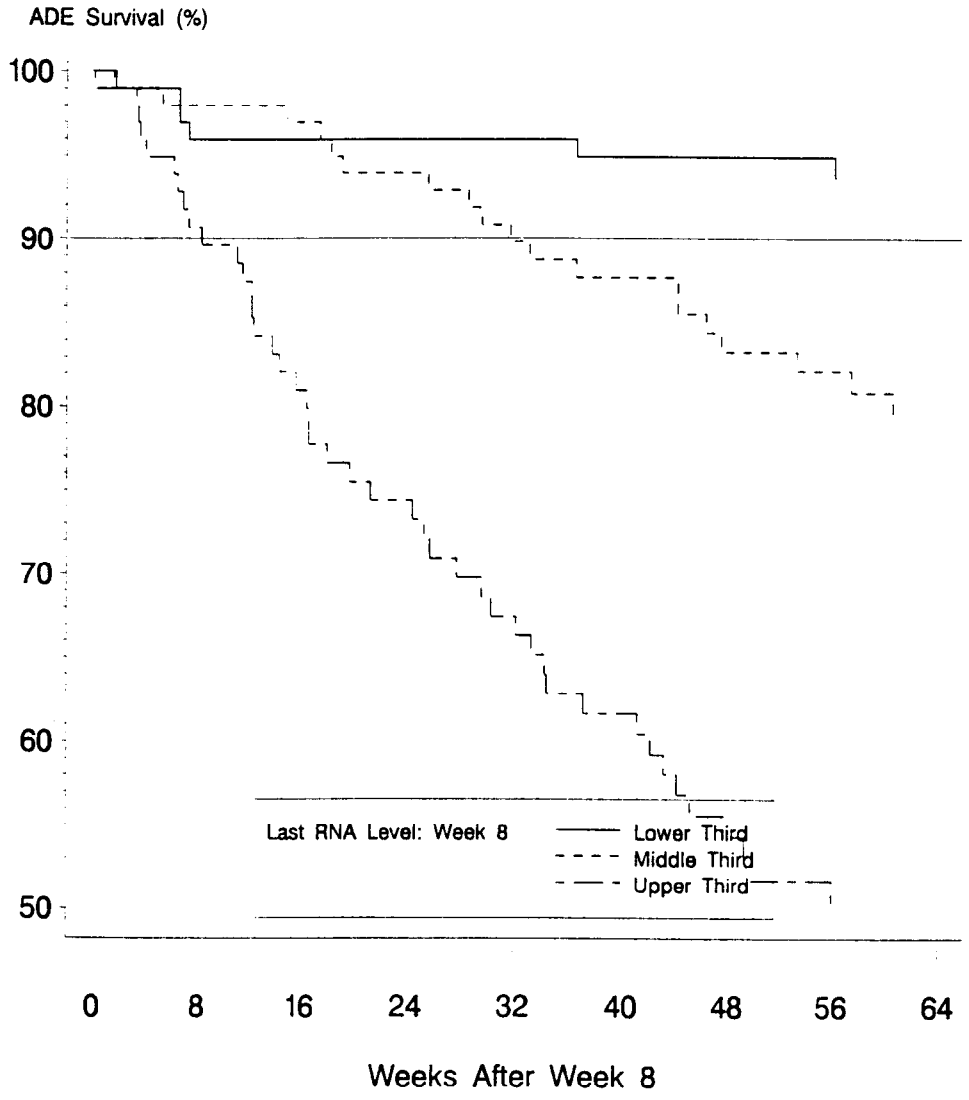


Figure 9
Kaplan Meier Estimated Survivals
Study NV14256 Treatment Group: ddC + SAQ 600mg
Patients Surviving ADE Free Through Week 8

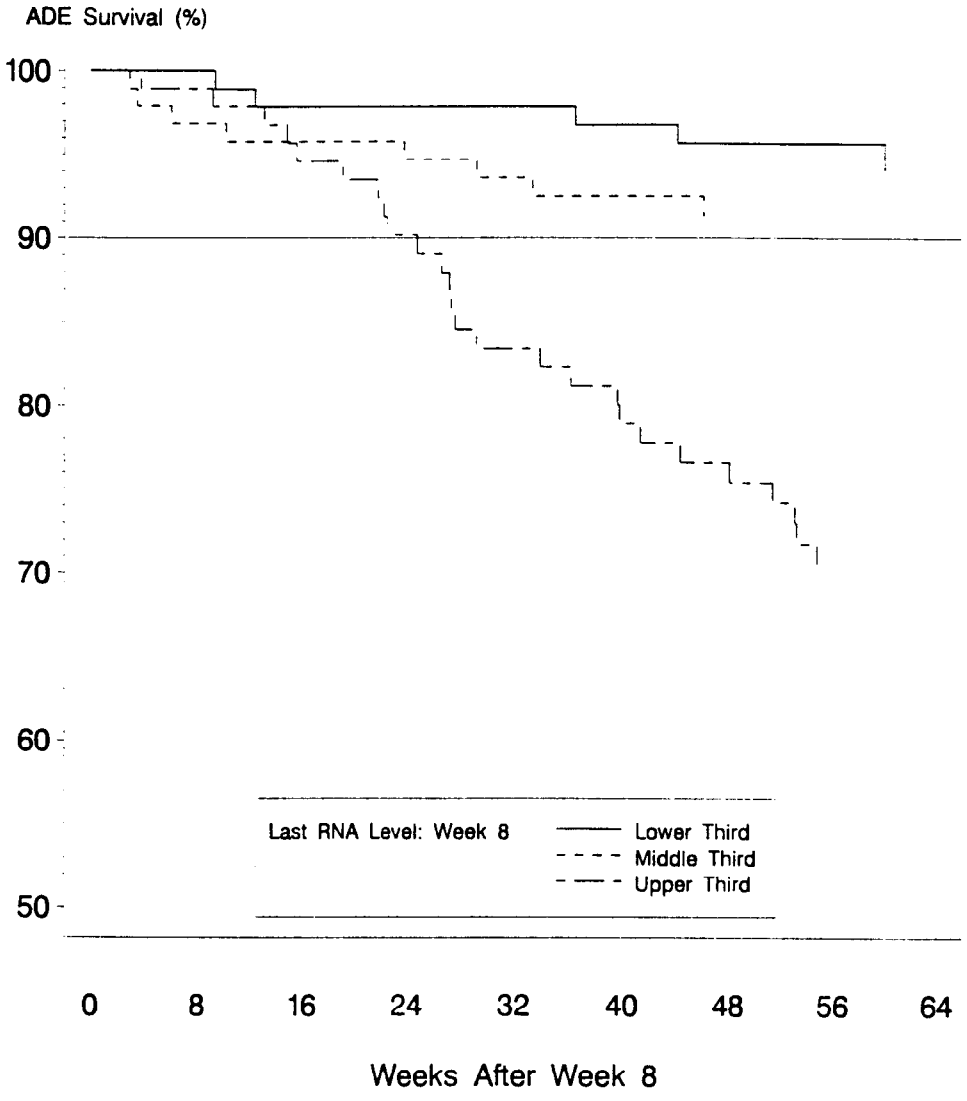


Figure 10
Hazard Ratio for Time to First ADE
Patients Surviving ADE Free Through Week 4
Study NV14256

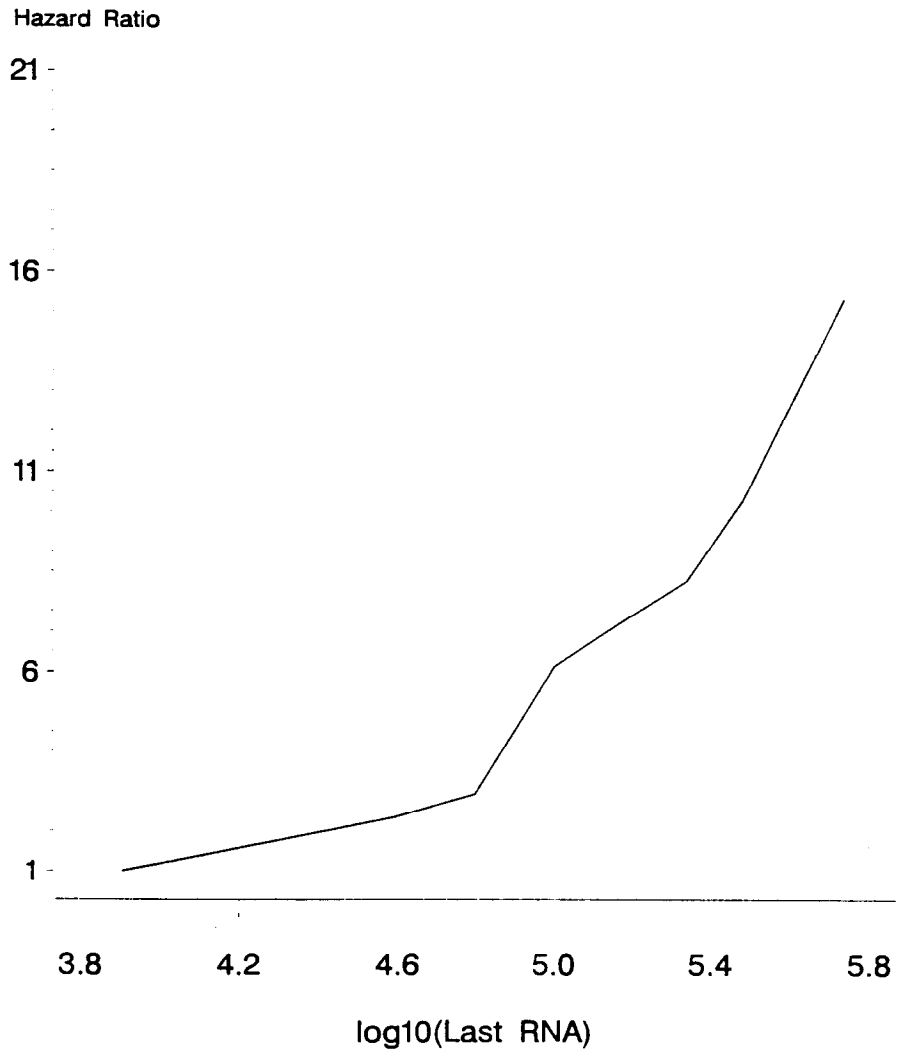
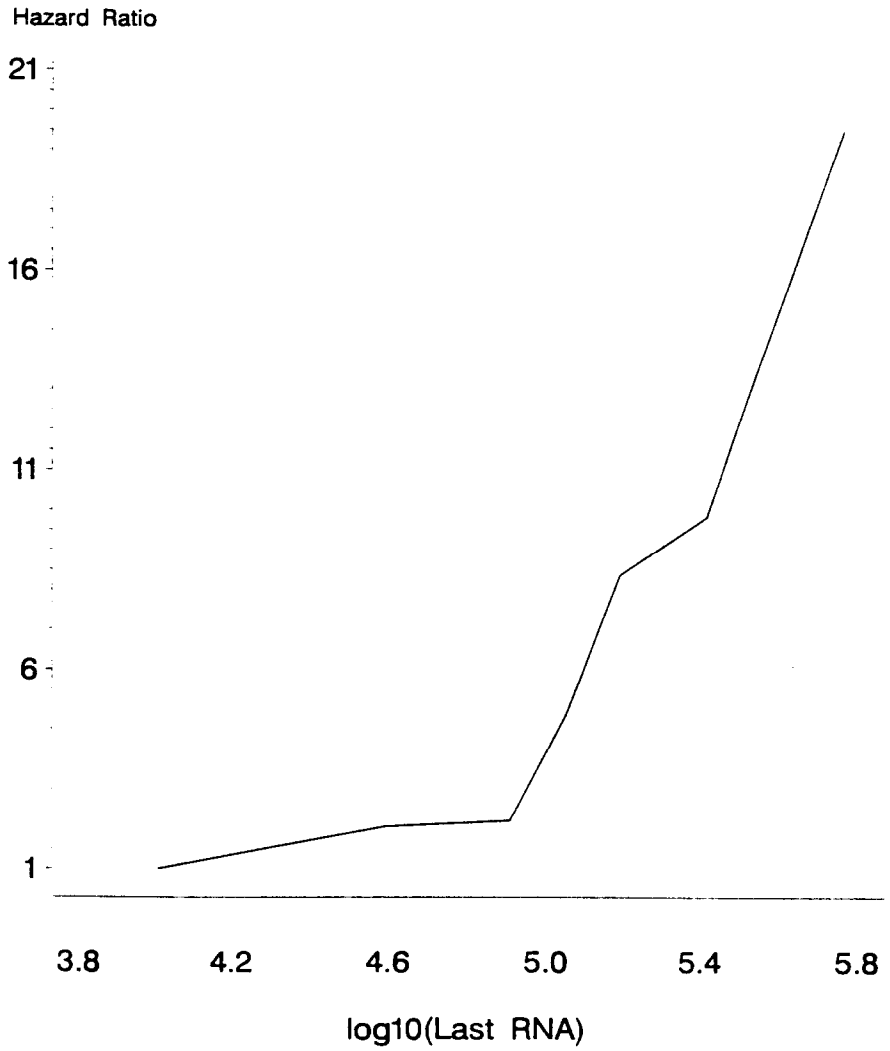


Figure 11
Hazard Ratio for Time to First ADE
Patients Surviving ADE Free Through Week 16
Study NV14256



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**2/99-RA**

# ATTACHMENT 21

# Misdiagnosis of HIV Infection by HIV-1 Plasma Viral Load Testing: A Case Series

Josiah D. Rich, MD, MPH; Nathan A. Merriman, ScB; Eleftherios Mylonakis, MD; Thomas C. Greenough, MD; Timothy P. Flanigan, MD; Brian J. Mady, MD; and Charles C.J. Carpenter, MD

**Background:** The availability of sensitive assays for plasma HIV viral load and the trend toward earlier and more aggressive treatment of HIV infection has led to the inappropriate use of these assays as primary tools for the diagnosis of acute HIV infection.

**Objective:** To describe limitations in the use of plasma viral load testing for the diagnosis of HIV infection.

**Design:** Case series.

**Setting:** Academic medical centers in Providence, Rhode Island, and Worcester, Massachusetts.

**Patients:** Three persons in whom HIV infection was falsely diagnosed by plasma viral load testing.

**Measurements:** Laboratory measures and clinical outcomes.

**Results:** Two cases of false-positive results obtained by using branched-chain DNA plasma viral load assays and one case of a false-positive result obtained by using reverse transcriptase–polymerase chain reaction plasma viral load assay are reported. All three plasma viral load tests yielded positive results with low values (1254 copies/mL, 1574 copies/mL, and 1300 copies/mL). Infection with HIV was initially diagnosed in all three patients, but each patient subsequently tested negative by HIV-1 enzyme-linked immunosorbent assay and repeated plasma viral load testing.

**Conclusion:** Physicians should exercise caution when using plasma viral load assays to detect primary HIV infection, particularly when the pretest probability of infection is low.

This paper is also available at http://www.acponline.org.

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The current standard of care for patients infected with HIV includes plasma viral load tests to monitor the effectiveness of antiretroviral regimens (1, 2). The assays approved for this use detect cell-free plasma viral RNA by using various amplification techniques (2). Access to these sensitive techniques and the trend toward earlier and more aggressive treatment approaches have led to the use of these assays as primary tools for the diagnosis of acute HIV infection (3, 4). Plasma viral load tests for HIV-1 were neither developed nor evaluated for the diagnosis of HIV infection; therefore, their diagnostic specificity is not well delineated when applied to persons who are negative for HIV antibody (5). We report two cases of false-positive results obtained by using branched-chain DNA assay (Chiron Quantiplex, Emeryville, California) and one case of a false-positive result obtained by using HIV reverse transcriptase polymerase chain reaction (RT-PCR) (Roche Amplicor Monitor, Basel, Switzerland) plasma viral load assay. These three cases demonstrate the potential problems of using HIV-1 plasma viral load tests for diagnosis of HIV infection.

## Case One

A previously healthy 12-year-old boy, whose HIV-infected mother is cared for at one of our institutions, presented for evaluation of a positive plasma viral load of 1254 copies/mL determined by using the branched-chain DNA assay (Chiron Quantiplex) for HIV-1 RNA. The patient's mother had received a diagnosis of HIV infection around the time of his birth, and the patient had tested negative for HIV-1 by enzyme-linked immunosorbent assay (ELISA) several times in the years after his birth. Although the patient reported no risk factors for HIV infection, he underwent plasma viral load testing after his primary care physician noted a skin lesion that was interpreted as herpes zoster. At our institution, the patient subsequently had a negative result on HIV-1 ELISA, a normal CD4 cell count and CD4:CD8 ratio, and a negative plasma viral load (also determined by using the branched-chain DNA assay). His skin lesion was diagnosed as impetigo, and he remains in excellent health 3 months after his initial presentation.

## Case Two

A previously healthy, pregnant 40-year-old woman presented for an HIV test. Her male sexual partner, with whom she had recently had repeated unprotected vaginal intercourse, had been given a diagnosis of HIV infection 1 week before her office visit.

The patient tested negative for HIV-1 antibody on the oral mucosal transudate (OraSure, Epitope, Inc., Beaverton, Oregon) HIV-1 oral specimen collection device but continued to be concerned about her HIV status. One week after her initial presentation, she underwent a plasma viral load test (Chiron Quantiplex) for HIV-1 RNA that yielded a positive value of 1574 copies/mL. The patient was told that she was probably infected with HIV. During the next 3 months, she had a negative result on an HIV-1 ELISA, a normal CD4 cell count and CD4:CD8 ratio, and three HIV-1 plasma viral load tests (all done by using branched-chain DNA assay) that showed an undetectable viral load. When the patient delivered a healthy baby 7 months after her initial presentation, another HIV-1 ELISA yielded negative results.

## Case Three

A 20-year-old healthy woman was referred for further evaluation by her primary care physician when she had a positive result on HIV-1 ELISA and an indeterminate result on a Western blot test. The patient's only risk factor was heterosexual intercourse, but she stated that her partner had used condoms consistently during the previous year. During a 4-month period after her indeterminate result on the Western blot test, she had a positive result on ELISA and an indeterminate result on a Western blot test on separate occasions. Five months later, both ELISA and a Western blot test yielded negative results, but the patient had a plasma viral load of 1300 copies/mL (determined by using RT-PCR assay [Roche Amplicor Monitor]). She was subsequently counseled that she was probably infected with HIV. Nearly 6 months after her initial indeterminate HIV test result, she was tested by a third laboratory and was negative for HIV-1 antibodies on both ELISA and Western blot test. She had a normal CD4 cell count and CD4:CD8 ratio and a plasma viral load that was undetectable on RT-PCR assay (Roche Amplicor Monitor). She remains healthy 8 months after her initial presentation.

## Discussion

These three cases, which were observed in one region during a 2-month period, are probably examples of false-positive results on HIV-1 plasma viral load tests. Only one other case of a false-positive HIV-1 plasma viral load has been fully documented; that test had been performed by using RT-PCR, and the result was thought to be related to the administration of an HIV-1 vaccine (6). The patients described here had normal CD4 cell counts and CD4:CD8 ratios, low plasma viral loads, and subsequent negative results on HIV-1 ELISA and plasma viral load tests. To our knowledge, the lowest reported plasma viral load during seroconversion is more than 17 times higher than the highest viral load detected in our three patients (7). Although transient HIV infection has been reported in infants, it is unlikely in two of our patients because they had not recently been exposed to HIV (8, 9). Other potential explanations of false-positive HIV-1 plasma viral load include laboratory error, cross-contamination, and mix-up of specimens. From the patient's perspective, false-positive results on an HIV test are potentially devastating, regardless of the cause. Further clinical experience is required to determine whether specific clinical circumstances correlate with an increased incidence of false-positive HIV-1 plasma viral load results.

The current standard diagnostic protocol for HIV infection is based on detection of HIV-1–specific antibodies. The combination of screening ELISA followed by a confirmatory Western blot assay has been more than 99% accurate in detecting HIV infection (10, 11). This protocol has a relatively low rate of false-positive results (approximately 0.0006%) but can have negative or indeterminate results during the 3 to 4 weeks before seroconversion (12–14). Although host antibody responses may be undetectable during this acute infection period, the viral load in plasma is usually very high and initial viremia usually occurs in 4 to 11 days (4, 7, 15, 16). The occurrence of high levels of viremia during primary HIV infection has led some physicians to use plasma viral load assays as diagnostic tests to detect early HIV infection. However, plasma viral load assays are designed for monitoring the effectiveness of antiretroviral therapies and for measuring the quantity of virus in patients with confirmed HIV infection, not for the diagnosis of HIV infection. Their performance in patients who are not infected with HIV is unknown (1, 2).

The first case illustrates the importance of following the most recent testing protocol for the diagnosis of HIV infection. The patient's pediatrician requested a plasma viral load assay because the patient, whose mother has asymptomatic HIV infection, presented with a skin rash thought to be consistent with herpes zoster. In this case, because primary HIV infection was not suspected, an HIV-1 ELISA should have been ordered and, if reactive, followed by a Western blot assay. In the second case, a plasma viral load assay was ordered despite a negative result on an oral mucosal transudate test (OraSure) because the patient was pregnant and was at substantial risk for recent exposure to HIV.

However, on the basis of current knowledge about viral replication during primary HIV infection, the patient's plasma viral load would probably have been much higher if she had been infected with HIV in the previous 2 weeks (15). In the third case and in other cases described in the literature, plasma viral load testing was used to further analyze an indeterminate result on an HIV-1 Western blot assay (3).

To minimize the occurrence of false-positive results, testing protocols for the diagnosis of HIV infection should include tests that complement each other. The HIV-1 ELISA assay, which has excellent diagnostic sensitivity, remains an important, inexpensive screening tool. Because of its high specificity, the HIV-1 Western blot assay is a reliable confirmatory test after reactive ELISA. Only patients who have a high pretest probability of a positive result should be evaluated for primary HIV infection by using plasma viral load testing. Such patients include those who are at high risk for recent exposure to HIV and present with indeterminate or negative results on Western blot tests and especially those with an appropriate accompanying clinical syndrome (4). A patient with a high HIV-1 plasma viral load is most likely in the process of seroconversion; although it is theoretically possible that a patient with an undetectable or low plasma viral load may have recently been infected with HIV, that possibility is much less likely.

It is important to consider the pretest likelihood of acute infection when counseling patients with negative results on serologic testing and a low plasma viral load. Physicians should explain that the patient may not be infected with HIV-1 but should take precautions to avoid infecting others until follow-up testing provides a definite result. If HIV-1 plasma viral load testing is used in the diagnosis of primary HIV infection before the development of serum antibodies, low positive plasma viral load results should be interpreted with caution and the patient's true disease status should be confirmed with repeated plasma viral load testing and follow-up serologic testing.

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